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FROM
THE ROCKEFELLER INSTITUTE
FOR MEDICAL RESEARCH

REPRINTS
VOLUME XXIX



NEW YORK
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1918

The Journal of Experimental Medicine

EDITED BY

SIMON FLEXNER, M.D.

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The Journal of Biological Chemistry

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THE PROPHYLACTIC AND THERAPEUTIC PROPERTIES OF THE ANTITOXIN FOR *BACILLUS WELCHII*.

By CARROLL G. BULL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 1, 1917.)

In a recent article¹ experiments were given which seemed to establish the following points: (1) under certain defined conditions, a bacteria-free toxic substance can be obtained from cultures of *Bacillus welchii*; (2) this substance possesses the physical properties of an exotoxin and, on animal inoculation, is capable of producing all the essential lesions and effects of infection with the bacilli; (3) animals which have received a number of graded doses of the toxin yield an immune serum which neutralizes *in vitro* all the pathologic effects of the toxin and exhibits power to prevent and control infections with both the spore and vegetative forms of the bacilli. These experimental results appeared to justify the conclusion that *Bacillus welchii* should be classed with *Bacillus diphtheriæ* and *Bacillus tetani* as a toxin-producing organism and that infections with the organism might be successfully combated by means of a specific immune serum. The present paper deals with more extensive and systematic experiments on the preventive and curative powers of the antitoxin.

Prophylaxis against Intoxication.

A series of guinea pigs ranging in weight from 500 to 800 gm. was given a prophylactic dose of antitoxic serum. Each animal received 0.25 cc. of the antitoxin² for each 100 gm. of weight, and the susceptibility of the prophylactic series to intravenous injections of the toxin

¹ Bull, C. G., and Pritchett, I. W., *J. Exp. Med.*, 1917, xxvi, 119.

² This antitoxin has now been prepared in the horse. The method employed for producing it will be the subject of a later paper, as well as the question of standardization. At present dosage is given in numbers of cubic centimeters of the serum employed.

was compared with that of normal guinea pigs at different intervals after the immune serum had been given. The following protocols give the results:

Experiment 1. 2 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 1, weight 510 gm., had received 1.5 cc. of the antitoxin subcutaneously. 10 cc. of toxin were injected into the jugular vein. No symptoms had developed 6 hours after the injection, but the guinea pig was found dead 20 hours after receiving the toxin. The autopsy showed lesions characteristic of *B. welchii* toxin.³

Guinea Pig 2, weight 660 gm., had received 1.6 cc. of antitoxin subcutaneously. 8 cc. of toxin were injected into the jugular vein. The guinea pig developed no symptoms and was discarded in perfect condition 3 weeks later.

Guinea Pig 3, weight 560 gm., normal control. 0.3 cc. of toxin was injected into the jugular vein. This guinea pig was found dead 16 hours later. Characteristic lesions were found at autopsy.

Experiment 2. 5 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 4, weight 660 gm., had received 1.6 cc. of antitoxin subcutaneously. 4 cc. of toxin were injected into the jugular vein. This animal developed symptoms of intoxication 2 hours after the injection and died at the expiration of 4 hours. The autopsy findings were characteristic.

Guinea Pig 5, weight 510 gm., had received 1.5 cc. of antitoxin subcutaneously. 2 cc. of toxin were given intravenously. No immediate or delayed symptoms arose, and the guinea pig was in perfect health 3 weeks later.

Guinea Pig 6, weight 770 gm., normal control. 0.25 cc. of toxin was injected into the jugular vein. The guinea pig was found dead 16 hours later. The autopsy findings were typical.

Experiment 3. 7 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 7, weight 670 gm., had received 1.7 cc. of antitoxin subcutaneously. 2 cc. of toxin were given intravenously. No symptoms developed, and the guinea pig was normal 3 weeks later.

Guinea Pig 8, weight 600 gm., normal control. 1 cc. of toxin was injected into the jugular vein. The guinea pig died $3\frac{1}{2}$ hours later, and characteristic lesions were present.

Experiment 4. 9 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 9, weight 650 gm., had received 1.6 cc. of antitoxin subcutaneously. 2 cc. of toxin were given intravenously. No symptoms had appeared 6 hours later, but the guinea pig was found dead 24 hours after the toxin was given. The autopsy findings were characteristic of *B. welchii* intoxication.

Guinea Pig 10, weight 650 gm., had received 1.6 cc. of antitoxin subcutaneously. 1.5 cc. of toxin were injected into the jugular vein. No symptoms of intoxication arose, and the guinea pig remained normal.

³ Differences in the manner of action of *B. welchii* toxin when administered by intravenous and subcutaneous routes are described in the paper already referred to.¹

Guinea Pig 11, weight 700 gm., normal control. 0.7 cc. of toxin was given intravenously. Death occurred 4 hours later, and the lesions were typical.

Experiment 5. 12 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 12, weight 640 gm., had received 1.6 cc. of antitoxin subcutaneously. 0.7 cc. of toxin was injected into the jugular vein. No symptoms had arisen 8 hours later. The guinea pig was found dead 22 hours after the toxin was given, and the autopsy findings were typical.

Guinea Pig 13, weight 660 gm., had received 1.7 cc. of antitoxin subcutaneously. 0.5 cc. of toxin was injected into the jugular vein. The guinea pig remained normal.

Guinea Pig 14, weight 600 gm., normal control. 0.28 cc. of toxin was given intravenously. This guinea pig was found dead 20 hours later. The autopsy findings were typical.

Experiment 6. 14 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 15, weight 570 gm., had received 1.5 cc. of antitoxin subcutaneously. 0.25 cc. of toxin was injected into the jugular vein. Death occurred 20 hours later. Characteristic toxin lesions were found at autopsy.

Guinea Pig 16, weight 580 gm., had received 1.5 cc. of antitoxin subcutaneously. 0.25 cc. of toxin was given intravenously. No symptoms developed, and the guinea pig was in perfect health 3 weeks later.

Guinea Pig 17, weight 600 gm., normal control. 0.27 cc. of toxin was injected into the jugular vein. The animal was found dead 20 hours after the toxin was given, and typical lesions were present.

The foregoing experiments show that a passive immunity to *Bacillus welchii* toxin can be conferred on guinea pigs by the administration of the antitoxin. In these instances the immunity persisted for about 2 weeks. The protection from the toxin was at first very pronounced, thirty-two acutely lethal doses being harmless when injected directly into the blood stream (Experiment 1, Guinea Pig 2) 2 days after the antitoxin was given. 5 days after the prophylactic administration of the antitoxin, eight lethal doses were still harmless (Experiment 2, Guinea Pig 5), but sixteen lethal doses killed. The immunity to the toxin gradually decreased and was about exhausted at the end of 2 weeks. At this time some of the prophylactic animals were still somewhat more resistant to the toxin than normal animals, while others manifested no increased resistance (Experiment 6, Guinea Pigs 15, 16, and 17).

The fact that animals can be passively immunized to the toxin may in itself be of considerable practical significance; but when it is remembered that the toxin is a powerful aggressin, preparing the field

for bacterial multiplication, its high significance becomes at once apparent. The next series of experiments further emphasizes the importance of the part played by the toxin in infection by the bacilli and shows conclusively that large numbers of highly virulent bacilli are practically harmless when deprived of their toxin.

Prophylaxis against Infection.

A series of guinea pigs, ranging in weight from 250 to 350 gm., was given subcutaneously on the inner aspect of one hind leg 1 cc. of antitoxin per 100 gm. of weight. The resistance of the prophylactic series to infection with fresh cultures of the virulent bacilli was compared with that of normal guinea pigs of the same size. The protected guinea pigs were infected subcutaneously in the leg opposite to the one in which they had received the antitoxin. The quantity of culture given the individual animals was calculated on the basis of body weight, a certain fraction of a cubic centimeter being given for each 100 gm. of weight. The following protocols illustrate the results:

Experiment 7. 24 Hours after the Prophylactic Dose of Antitoxin.—Guinea Pig 18, weight 280 gm., had received 2.8 cc. of antitoxin. 0.28 cc. of culture was given subcutaneously. 7 hours later the infected leg was moderately swollen and stiff; no local crepitation and no symptoms of general intoxication. 24 hours after the inoculation the local swelling had almost disappeared, and the guinea pig was apparently well.

Guinea Pig 19, weight 280 gm., had received 2.8 cc. of antitoxin and was given 0.5 cc. of culture. 7 hours later the infected leg was swollen and stiff; crepitation could not be elicited, and there was no general intoxication. The next day the swelling and stiffness were subsiding, the guinea pig was active, and no extension of the infection arose.

Guinea Pig 20, weight 250 gm., had received 2.5 cc. of antitoxin and was given 0.9 cc. of culture. The results were identical with those of the two preceding animals.

Guinea Pig 21, weight 310 gm., normal control. 0.006 cc. of culture was given subcutaneously. This guinea pig was found dead 22 hours after it was inoculated. Autopsy revealed edema, gas, and disorganization of tissue, lesions typical of *B. welchii* infection.

In Experiment 7 it is shown that 50, 83, and 150 lethal doses of culture respectively failed to infect the guinea pigs which had re-

ceived a protective injection of antitoxin 24 hours previously. As 150 lethal doses was the largest quantity of culture given the height of the resistance to infection was not accurately determined.

Experiment 8. 3 Days after the Prophylactic Dose of Antitoxin.—Guinea Pig 22, weight 250 gm., had received 2.5 cc. of antitoxin and was given 0.4 cc. of culture. No symptoms developed aside from local swelling and stiffness which rapidly subsided. No evidence of multiplication of the bacilli.

Guinea Pig 23, weight 310 gm., had received 3.1 cc. of antitoxin. 0.6 cc. of culture was injected into the opposite leg. The symptoms and results were the same as in the preceding animal.

Guinea Pig 24, weight 300 gm., normal control. 0.004 cc. of culture was given subcutaneously. The animal died 22 hours later. Lesions of *B. welchii* infection were present at autopsy.

In this experiment the limit of the resistance to infection was again not reached, although one of the guinea pigs also received 150 lethal doses of the culture.

Experiment 9. 5 Days after the Antitoxin.—Guinea Pig 25, weight 260 gm., had received 2.6 cc. of antitoxin. 1.5 cc. of culture were injected into the opposite leg. Infection did not develop, and the local swelling and stiffness which always follow these inoculations rapidly subsided.

Guinea Pig 26, weight 280 gm., had received 2.8 cc. of antitoxin. 2 cc. of culture were given. Infection developed, and the guinea pig died 30 hours after inoculation. Lesions typical of *B. welchii* infection were present at autopsy.

Guinea Pig 27, weight 290 gm., normal control. 0.005 cc. of culture was injected subcutaneously. The guinea pig was found dead 20 hours later and showed typical lesions.

This experiment indicates that a protected guinea pig could resist 300 but not 400 lethal doses of the culture 5 days after the antitoxin had been given.

Experiment 10. 8 Days after the Antitoxin.—Guinea Pig 28, weight 260 gm., had received 2.6 cc. of antitoxin. 0.3 cc. of culture was given subcutaneously. The usual local swelling and stiffness followed, but no infection arose.

Guinea Pig 29, weight 280 gm., had received 2.8 cc. of antitoxin subcutaneously. 0.5 cc. of culture caused a fatal infection, with characteristic lesions.

Guinea Pig 30, weight 275 gm., normal control. 0.005 cc. of culture was given subcutaneously. Infection developed, and the guinea pig died 23 hours after inoculation.

This experiment shows that 60 lethal doses of culture failed to infect 8 days after the antitoxin had been administered. The resistance of the protected guinea pigs was tested again on the 11th day, and twenty lethal doses were not infectious. On the 14th and 15th days, however, the antitoxin-treated guinea pigs proved susceptible to infection, but whether to the same degree as the normal controls the experiment does not indicate. We may, however, assume that the antitoxin becomes greatly reduced in quantity from the 12th to 13th day after its administration.

Therapeutic Property of the Antitoxin.

We may now consider the effects which the antitoxin exerts upon established infection with *Bacillus welchii*. For this purpose guinea pigs weighing from 500 to 600 gm. were used, smaller ones being so susceptible to the infection that there is little time afforded for treatment. The amount of a standard culture that would infect and kill all of a series of guinea pigs between 30 and 48 hours was first determined. The infecting dose was calculated on the basis of body weight, and it was found that about 0.035 cc. of culture per 100 gm. of weight was effective within the time limit mentioned in all the animals inoculated ranging in weight from 500 to 600 gm. The procedure was to infect a number of animals at one time with the same culture and allow 24 hours to elapse before beginning treatment. At this time the condition of the animals was noted, especially the extent of the local lesions and the symptoms of general intoxication. One of the animals was etherized and autopsied, and to one or more the antitoxin was administered, while the others were left as untreated controls. Illustrative protocols follow:

Experiment 11.—Guinea Pig 31, weight 510 gm. 0.18 cc. of culture was injected into the muscles on the inner aspect of the hind leg. 24 hours after inoculation, a gaseous phlegmon involving the infected leg and the adjacent abdominal wall had developed, crepitation was readily elicited, and the guinea pig exhibited symptoms of severe intoxication—rough coat, rapid respiration, and drowsiness. 3 cc. of antitoxin were injected into the jugular vein. The next day the local lesion was subsiding, crepitation could not be elicited, the toxic symptoms had disappeared, and the guinea pig ate greedily. 3 cc. of antitoxin were given sub-

cutaneously to prevent a recrudescence of the infection. The general condition improved from day to day, while the infected leg became gangrenous, sloughed, and was infected with pyogenic organisms. Healing finally occurred, but the scar tissue caused deformity of the leg.

Guinea Pig 32, weight 560 gm. 0.2 cc. of culture was injected into the muscle on the inner aspect of the hind leg. 24 hours after inoculation there were swelling and edema, crepitation, and intoxication. The animal was not treated, and it died exactly 48 hours after inoculation. Autopsy revealed the characteristic lesions of *B. welchii* infection.

Guinea Pig 33, weight 580 gm. 0.2 cc. of culture was injected in the same manner as in Guinea Pigs 31 and 32. 24 hours after inoculation, the guinea pig was etherized and autopsied. There was a large gaseous phlegmon involving the infected leg and adjacent abdominal wall. The muscles of this region were pulpid and laden with bacilli. The opposite groin contained a gelatinous, serosanguineous exudate. The muscles of the abdominal wall and diaphragm were of pinkish hue. The lungs were pink and edematous.

Guinea Pig 34, weight 605 gm., was inoculated with 0.21 cc. of culture. This animal served as an untreated control and died at the end of 40 hours of typical *B. welchii* infection.

Experiment 11 was repeated a number of times, and it was found that the infection could be regularly arrested by the antitoxin after it was well established and extensive destruction of the tissues in the region of the infection had already taken place. Symptoms of severe general intoxication were always present at this stage of the disease. The effect of the antitoxin was often noticeable within from 30 minutes to 1 hour after its administration. The treated guinea pigs would become more active, their coats smooth, and they would be attracted by food, while the controls remained crouched in a corner, with heads down and coats rough, and could not be induced to eat.

DISCUSSION.

The experimental results here reported with the preventive and therapeutic applications of the antitoxin are highly suggestive. They derive significance from the fact that *Bacillus welchii* infections in guinea pigs and other susceptible animals⁴ are comparable with in-

⁴ Protection and curative experiments have been carried out also with the more highly susceptible pigeon. They confirm the results with guinea pigs with certain variations due to the difference in species.

infections with this organism in man. The experimental infections in the guinea pig differ, however, from the natural infection in man in two important points: (1) man possesses a higher natural resistance to infection; (2) the guinea pigs were infected with fresh virulent cultures, while man must, in the great majority of instances, derive infection from spores. Moreover, the protected guinea pigs were given many lethal doses of the living cultures. Such massive inoculations do not occur in man. It may therefore be safely predicted that man will not develop the infection as long as his body fluids and tissues contain adequate quantities of the antitoxin.

The possibilities of this passive serum protection has natural limits of time, depending upon the rapidity of elimination of the foreign serum. The experimental data presented in this paper, which agree with the experience with antidiphtheritic and antitetanic antitoxins, indicate that, in all probability, a passive immunity to *Bacillus welchii* infection of at least 2 weeks' duration can be conferred upon man by a single injection of the antitoxin. This immunity will be sufficient in the majority of instances, since only sporadic cases of *Bacillus welchii* infection arise later than the 10th day after injury, and the greater number occurs within 48 hours of that time.

In the light of the results obtained in treating the infection in guinea pigs, it is reasonable to hope that the antitoxin will be of value also as a therapeutic agent. The indications are that early infectious cases can be readily arrested and the more advanced and severe ones ameliorated, if not wholly checked, so that surgical interference may be resorted to with greater probability of effectiveness.

The antitoxin in man should be administered intravenously and probably locally, about the wound, as well.

SUMMARY.

1. It has been possible to confer on guinea pigs a passive immunity of about 2 weeks' duration to *Bacillus welchii* toxin through a protective administration of the antitoxin.

2. Guinea pigs which had received a prophylactic dose of *Bacillus welchii* antitoxin exhibited pronounced resistance to infection with the virulent bacilli for a period of 12 days.

3. Established infections in guinea pigs with *Bacillus welchii* have been arrested and controlled by treatment with the antitoxin.

4. The opinion has been expressed that it will be possible to prevent *Bacillus welchii* infection in man through the prophylactic use of the antitoxin and developed cases of the infection may be controlled by therapeutic injections of the same agent.

NOTES ON THE STANDARDIZATION AND ADMINISTRATION OF ANTIMENINGOCOCCIC SERUM.

BY HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Now that wide and repeated experience here and abroad with reliable preparations of the antimeningococcic serum in the treatment of epidemic meningitis has resulted in a consensus of opinion favorable to its value, it is time to take up seriously the question of providing federal standardization in order that the public may be protected from poor and worthless preparations.

Unfortunately, as the distressing English experience of 1915, later retrieved when supplies of potent serum became available, showed, the commercial producer of the serum cannot be left to determine his own method of manufacture and his own standard of potency.

There has been misconception also as to the practicability of standardization, which needs to be removed by a statement of the essential facts of the case.

Undoubtedly, our minds have been clouded and our action impeded by the notion, which is only slowly giving away, that the only therapeutically active serums are those which are antitoxic in the strict sense, while the other class of antibacterial serum stands on an insecure therapeutic foundation. In the face of the convincing data available relative to the effects of the antimeningococcic serum and the growing favorable testimony to the value of the antipneumococcic serum Type I, this prejudice is being overcome.

The essential obstacle in each case—that of the antimeningococcic and antipneumococcic serum—arises from the circumstance that neither the meningococcus nor the pneumococcus is a consistent species, both being separable into more or less distinct type groups. As regards the pneumococcus, the practical case is simple and the whole matter of standardization is reducible to a formula, since up to the present the serum Type I alone is therapeutically effective, and no

serum should be accepted as up to the standard which does not contain given quantities of antibodies for Type I pneumococcus.

While the case is not quite so simple for the meningococcus, it is by no means so complex as to defy ready solution. In one respect, the therapeutic possibilities of the antimeningococcic serum are far wider than serums for pneumococcus, since it can be made effective against all the meningococci causing epidemic meningitis.

Fortunately for our purpose, 80 per cent or more of all the cases of epidemic meningitis are caused by two type strains or groups of the meningococcus, regular or normal and parameningococcus, and nearly the remaining 20 per cent by two more strains not quite so definitely marked off biologically as the former or intermediate meningococcus A and B.¹ Hence, a potent serum may be prepared with, say, the four type cultures, which, if of sufficient titer, may be accepted as standard.

Any one familiar with the recent invaluable English experience which has been collected into a pamphlet issued by the British Medical Research Committee,² will know that, by pursuing a course similar to the one just outlined, they have insured supplies of an efficient preparation of the antimeningococcic serum, whereas formerly, without this control, they were grievously disappointed in the action of the available product.

This procedure will, as stated, yield a valuable and reliable preparation. In following it, there is nothing to prevent a more conscientious or ambitious manufacturer improving further his particular brand of serum by adding to the selected type cultures examples of the variants from the types which are occasionally encountered. This practice should indeed be encouraged; but it should not permit any clouding of the essential issue, which is to produce a highly potent serum for the standard cultures of meningococcus.

1. A comparative study might readily reveal that the four type groups here given correspond with the so-called Types I, II, III and IV of the English classification.

2. Bacteriological Studies in the Pathology and Preventive Control of Cerebro-spinal Fever Among the Forces During 1915 and 1916, Special Report Series, No. 3, National Health Insurance, Medical Research Committee, London, 1917.

It is my intention to define what would be a readily realizable and adequate standard of potency. But before doing this, I wish to describe a series of tests which were carried out very recently with commercial samples of the antimeningococcic serum and, coincidentally, with three other samples prepared by departments of health and the Rockefeller Institute.

Tests of Commercial Serums.

Several immunity reactions have been employed in testing the value of therapeutic serums. When applied to the antimeningococcic serum, they arrange themselves in the following order of specificity: (1) agglutination at 55 C.; (2) opsonization; (3) complement fixation; (4) anti-infectious power, and (5) antitoxic power. Gradually, agglutination of the type cultures of the meningococcus is displacing other and less indicative methods of standardization. Accurate clinical experience also is confirming this decision. We have made a series of observations, which extend now over two years, on the relation of agglutination to the therapeutic efficiency of the serum. Thus, we have tested the meningococci isolated from the cerebrospinal fluid against several so-called polyvalent samples of the serum; and we have noted that when the sample of serum failed to influence the course of the disease, agglutinating power was either absent or very low. Moreover, we have also noted that the replacement of the impotent preparation of serum with one carrying agglutinins for the culture in question would, as a rule, control the infection. A serum of proper standard should agglutinate the four type cultures mentioned in dilutions of from 1:400 to 1:1,000. The still more efficient serum may show agglutination for all the variants in dilution of from 1:200 to 1:500.

Five samples of antimeningococcic serum produced by the larger producers of biologic products were purchased in the open market. The dates for return on all the samples were 1918. Presumably, therefore, they were recent products. To them were added three other samples: two had been obtained not long before from departments of health, and the third was a recent sample of the Rockefeller Institute preparation. They were examined for physical properties and for agglutination against four type cultures. The results are given

in the accompanying table. The dilutions of the serum were set up by letters so that the worker carrying out the agglutination tests was not aware of the origin of the several samples.

Moreover, the physical properties of certain samples were such as to make their employment by intraspinal injection, to say the least, dangerous. To explain this point, the accompanying illustration was made.

Results of Agglutination Tests Made with

Sample	Date for Return on Label	Origin	Appearance	Regular or Normal Meningococci													
				Stock Culture No. 1							Stock Culture No. 7						
				100	200	400	800	1600	3200	6400	Control	100	200	400	800	1600	Control
A	7/22/18	Commercial	Cloudy, amber colored	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	1/27/18	Noncommercial	Clear, straw col- ored	++	++	-	-	-	-	-	-	++	++	+	-	-	-
C	11/ 2/18	Commercial	Clear, straw col- ored	+	+	-	-	-	-	-	-	++	+	-	-	-	-
D	6/20/18	Commercial	Dark, opaque, thick consistence	+	-	-	-	-	-	-	-	++	+	-	-	-	-
E	12/7/18	Commercial	Slightly cloudy, straw colored	-	-	-	-	-	-	-	-	++	+	-	-	-	-
F	7/25/18	Commercial	Slightly cloudy, straw colored	++	+	-	-	-	-	-	-	++	++	+	±	-	-
G	—	Rockefeller Institute	Clear, amber col- ored	++	++	±	-	-	-	-	-	++	++	+	-	-	-
H	11 22/17	Noncommercial	Clear, straw col- ored	++	-	-	-	-	-	-	-	++	++	++	-	-	-

* The agglutinations were conducted at 55 C. over night by the macroscopic method.

Summary of the Results of the Test.

Serum A (commercial) is slightly active against normal or regular culture No. 10 and sufficiently active against the parameningococcus culture No. 60. In other words, it is not sufficiently representative of the different type cultures to be suitable for therapeutic purposes. Moreover, the sample, while transparent, contains hemoglobin in excess and in an amount giving a red color to the serum. Severe reactions would follow its administration.

Serum B (board of health). The agglutinins are somewhat developed for the regular or normal meningococcus Nos. 1 and 7, well developed for the two irregular cultures Nos. 10 and 30, and for the parameningococcus. It is a useful product, but could be improved by the employment for immunization of larger quantities of the regular meningococcus cultures.

Serum C (commercial). Agglutinins are present in doubtful or small amounts for the regular meningococcus Nos. 1 and 7, and in somewhat larger quantity for the irregular culture No. 10 and the parameningococcus. This serum, which ful-

*Different Antimeningococcic Serums.**

Parameningococcus								Intermediate Meningococci													
Stock Culture No. 60								Stock Culture No. 10							Stock Culture No. 30						
100	200	400	800	1600	3200	6400	Control	100	200	400	800	1600	Control	100	200	400	800	1600	3200	6400	Control
++	++	++	++	±	-	-	-	+	±	-	-	-	-	±	-	-	-	-	-	-	-
++	++	++	+	±	-	-	-	++	++	++	+	±	-	++	++	++	++	-	-	-	-
++	+	±	-	-	-	-	-	++	++	++	+	-	-	++	+	-	-	-	-	-	-
++	++	-	-	-	-	-	-	++	++	++	+	±	-	++	-	-	-	-	-	-	-
++	++	+	±	-	-	-	-	++	++	++	++	++	-	++	++	-	-	-	-	-	-
++	++	-	-	-	-	-	-	++	++	++	++	++	-	++	++	+	-	-	-	-	-
++	++	++	++	++	++	++	-	++	++	++	++	++	-	++	++	++	++	+	-	-	-
++	++	++	+	-	-	-	-	++	++	++	++	+	-	++	++	+	-	-	-	-	-

fills the requirements in physical condition, is of uniformly low titer and improperly balanced in type agglutinins. Its therapeutic efficiency is dubious.

Serum D (commercial). The physical properties of this sample (as shown in the illustration) condemn it wholly. It consists of a dark, opaque, rather heavy liquid, which did not clarify on centrifugalization for twenty minutes at 3,500 speed. The preparation would undoubtedly give rise to severe, possibly to dangerous reaction. And yet the product is marketed in dark amber glass so that the physician would not detect the color and suspect the danger. In order to be fair, it should be stated that another sample obtained directly from the New York office of the firm was straw colored and clear; but there is no excuse for putting

out the former preparation. Had the container been clear glass, the sample would have been rejected; hence until clear white glass is used, it is advisable to pour out or inject about 1 c.c. of the serum into a test tube or other receptacle before administering it. Moreover, the agglutinins are present in low quantity except for irregular type culture No. 10. Aside from the physical properties of the one specimen, the preparation is unsatisfactory for therapeutic purposes.

Serum E (commercial). Agglutinins are present in adequate quantity for irregular culture No. 10 and for the parameningococcus culture. The quantity present for the regular meningococcus is minimal. The preparation is unsatisfactory for therapeutic purposes.

Serum F (commercial) represents the regular meningococcus and parameningococcus and the two irregular type cultures, but is improperly balanced. Taken as a whole, its agglutination titer is too long.

Serum G (Rockefeller Institute) represents adequately all the type cultures. A balanced serum which should fulfil therapeutic expectations.

Serum H (board of health) is a satisfactory product that should be therapeutically active.

COMMENT.

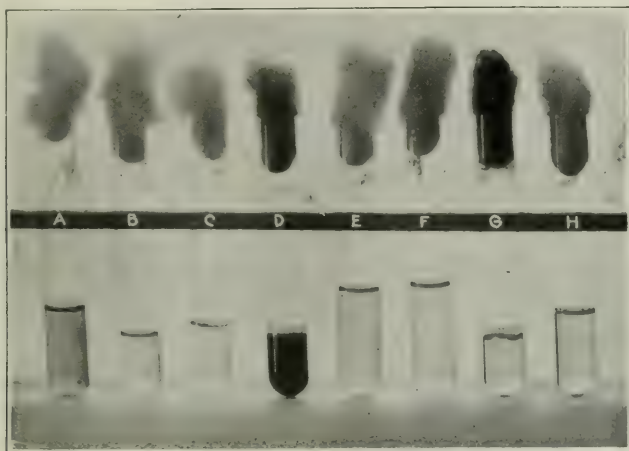
A critical examination of the results of the tests exhibited in the table brings out points both interesting and practically of high importance.

The first thing that impresses one is the striking inferiority of the commercial as compared with the noncommercial products. There would appear to be very little excuse for this discrepancy. It is particularly true of the antimeningococcic serum that it is unsafe to reduce its preparation to mere routine. Second bleedings without further injections of cultures should not be made, and in no instance should the horses be bled for serum until a test bleeding has shown that agglutinins are present in adequate amount for the principal type cultures. The practice of mixing samples of serums of high and low titer to increase the volume collectable should never be practiced.

There is nothing involved in the preparation of a satisfactory serum that cannot be mastered in a properly organized commercial laboratory. But knowledge of the subject, conscientiousness and vigilance are demanded to obtain a product that will fulfil reasonable standard conditions and be therapeutically effective. It is to be trusted that merely by pointing out present deficiencies, rectification will be made; and it may also be hoped that the constituted federal authorities will

establish and enforce a standard which is readily attainable and secure.

There is one point of technical interest in the table. Comparison of the agglutinability of regular or normal cultures Nos. 1 and 3 shows that one is more readily agglutinable than the other. This is a familiar phenomenon and one recognized in respect to many bacterial species. Its practical import in this connection is to the effect that in controlling the agglutination titer, reliance is not to be placed merely on a highly agglutinable culture.



Specimens of antimeningococcic serums illustrating variations in the physical condition of commercial products.

There is no difficulty involved in this procedure, since it will practically never happen, and indeed it should not be permitted to conduct the immunization with a single regular or a single parameningococcus culture. Several of each, together with the representatives of the intermediate types, should properly be employed for injection.

It will be sufficient and a great gain to have progressed so far. As already indicated, a still more perfect serum product can be turned out by paying constant attention to the rarer variants and introducing them into the scheme of immunization, controlling their effects also by agglutination tests. But this refinement is one not to be introduced into the plan of standardization.

Standardization.

The next question relates to a practical standard for the serum. It should include two requirements. The first should define the physical qualities of the product which are acceptable. This definition should include absence of more than a trace of hemoglobin, color of straw yellow to amber, perfect clearness, or if slightly turbid, clearing on standing for twelve hours. As regards the preservative, tricresol is to be preferred, and the strength should not exceed 0.35 per cent and may safely be reduced to 0.2 per cent, provided due care is exercised in collecting and bottling the serum.

Next, the employment of dark glass containers should be prohibited. The container, whether bottle or syringe, should be of clear white glass, and the labels arranged so as to permit of inspection of the contents from without. The containers should be wrapped in blue paper or otherwise enclosed so as to exclude the actinic rays of light.

Finally, the agglutination titer for each of the four type cultures should be from 1:400 to 1:1,000, as determined by the macroscopic method after incubation at 55 C. for sixteen hours (over night).

There should perhaps be added one more comment. With the assembling of large numbers of recruits, it is quite probable that epidemic meningitis may become more widely and numerously prevalent in this country. In the interests, therefore, of the military and civil populations, the supplies of the antimeningococcic serum now so generally employed in treatment should be vigorously controlled.

Administration.

As the result of a somewhat extensive experience with the clinical administration of the serum, I wish to direct attention to a few points of value. Whether or not the serum is to be effective depends in the first instance on its content in immunity bodies; but it depends also on the dosage, early administration and the frequency of repetition. The question of dosage has been considered in Dr. Flexner's³ recent paper and need not be discussed here. It remains to be said that

3. Flexner, S.: Mode of Infection, Means of Prevention, and Specific Treatment of Epidemic Meningitis, *THE JOURNAL A. M. A.*, Aug. 25, 1917, p. 639; Sept. 1, 1917, p. 721; Sept. 8, 1917, p. 817.

while the temperature remains high and meningococci are still present in the cerebrospinal fluid, injections every twelve hours, except in very young babies, should be resorted to unless clinical indications to the contrary exist. The next interval between injections should be twenty-four hours, then forty-eight hours. Subsidence of high temperature, clearing of cerebrospinal fluid with disappearance of the meningococcus, and general improvement in the condition of the patient, are the indexes for moderating the energy of the treatment.

The position of the patient is of moment. In order to distribute the serum over the surface of the brain and into the lateral ventricles after the intraspinal injection, the foot of the bed should be raised from 8 to 12 inches and kept so for six hours if possible. Sometimes the headache resulting from this position may make it expedient to return the bed to the normal position. At the end of six hours, the foot of the bed is lowered to its original position, and the other end raised until the time for the next lumbar puncture. During this time, the turbid fluid or pus is collected in the lower part of the spinal canal to be drawn off at the next puncture.

In addition to treating all cases by intraspinal injection of the serum, it is advisable to administer one or more doses intravenously in fulminant or very severe cases, or cases in which there are numerous skin hemorrhages. The quantity thus injected should be from 50 to 100 c.c., depending on the age of the patient and the severity of the infection. The usual precautions for guarding against anaphylactic reaction should of course be taken. Intravenous injection has been employed also to supplement the intraspinal ones in cases of less severity coming late under the serum treatment. Whether this is a real advantage cannot be stated positively; in some instances, it appeared to be beneficial. Obviously the chances of recrudescence and of blocking off of the ventricles will be lessened.

Ventricular puncture and injection of serum into both lateral ventricles should not be delayed when symptoms of cranial involvement remain after the spinal fluid is almost normal.

CARRIAGE OF THE VIRUS OF POLIOMYELITIS, WITH SUBSEQUENT DEVELOPMENT OF THE INFECTION.

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PLATES 55 AND 56.

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The solution of the problem of the mode of infection in poliomyelitis has been attempted in various ways, with results which have led to the conclusion that the microbic cause is conveyed from one individual to another by personal contact. This belief is based upon clinical observation and experiment. Wickman first brought clinical proof, since supported by many independent observations, of the correctness of this generalization; and Flexner and Lewis, and later Kling and Pettersson, provided the experimental demonstration of its adequacy.

However, a considerable number of physicians and others still refuse to accept this explanation. They hold that the mode of infection remains undiscovered, or they account for it through some variety of insect transmission, also undetected. In recognition of the skepticism still prevailing, we have been led to describe in detail the experimental demonstrations of the carriage by healthy persons of the virus of poliomyelitis, to which may now be added our own successful inoculations. Our results include the demonstration, recorded for the first time, that a proved carrier of the virus may come down with acute poliomyelitis. This observation should serve to strengthen the position of those who accept as established the personal communication of the microbic cause, or virus, of the disease.

* Maintained by a special fund privately donated.

Previous Reports.

Wickman's¹ clinical studies may be said to have disseminated the view of the personal factor in the communication of the virus of poliomyelitis. He emphasized the occurrence and epidemiological importance of the non-paralytic or abortive cases, the first description of which is usually credited to him, and of healthy intermediaries, or bacillary carriers, who function as purveyors of the microbic agent. His study constituted a great step forward; but the first person to allude to non-paralytic cases of epidemic poliomyelitis is Caverly,² who records the occurrence of 6 cases among the total of 132 cases on which he based his report describing the Rutland epidemic of 1894.

Soon after Landsteiner and Popper's³ experimental transmission of poliomyelitis, Flexner and Lewis⁴ detected the virus in the nasopharyngeal mucous membrane of infected monkeys. This observation, soon confirmed by several independent bacteriologists, was followed by a study made by Kling, Pettersson, and Wernstedt⁵ who injected into monkeys buccal washings from so called abortive cases and from healthy contacts. Their results were inconclusive, as the clinical condition produced was not typical of poliomyelitis, and the pathological changes described as present in the spinal cord were not characteristic of the disease. They explained the discrepancy by the supposition that the virus present in the abortive cases and healthy carriers was relatively avirulent. This view is repeated in their recent report⁶ in which they describe an instance of healthy carriage of the highly active virus inducing paralysis and characteristic lesions. The first demonstration of the typical virus in the nasopharyngeal washings of healthy persons was, however, made by Flexner, Clark, and Fraser,⁷ whose report follows in detail.

E. A., female, age 4 years and 4 months. The patient had been ill from Oct. 12 to 17, 1912. On the latter date she was admitted to the Hospital of The Rockefeller Institute for Medical Research, suffering from severe paralytic polio-

¹ Wickman, I., Beiträge zur Kenntnis der Heine-Medinschen Krankheit, Berlin, 1907.

² In view of the importance which the non-paralytic cases have assumed in the epidemiology of poliomyelitis it is pertinent to quote Caverly, who states that paralysis occurred in 119 cases, 7 cases died before paralysis was detected, "and the remaining 6 had no paralysis, but all had a group of symptoms very common in the initial stage in those which were paralyzed, such as headache, fever, convulsions, or nausea, one or all" (*J. Am. Med. Assn.*, 1896, xxvi, 1).

³ Landsteiner, K., and Popper, E., *Z. Immunitätsforsch., Orig.*, 1909, ii, 377.

⁴ Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 1140.

⁵ Kling, C., Pettersson, A., and Wernstedt, W., *Communications Inst. méd. État à Stockholm*, 1912, iii, 5.

⁶ Kling, C., and Pettersson, A., *Deutsch. med. Woch.*, 1914, xl, 320.

⁷ Flexner, S., Clark, P. F., and Fraser, F. R., *J. Am. Med. Assn.*, 1913, lx, 201.

myelitis. She subsequently improved and was discharged. Oct. 28. The mother and father of the child were subjected to a nasopharyngeal irrigation with normal saline solution; about 150 cc. of washings were obtained. The fluid was shaken and passed through a Berkefeld filter; of the filtrate, 1.5 cc. were injected the same day into the sheath of each sciatic nerve and 140 cc. into the peritoneal cavity of a *Macacus cynomolgus* (Monkey A). Recovery from the anesthesia was prompt and the animal remained well until Nov. 11, when it was noted to be excited and to drag the right leg; the left leg was weak. Nov. 12. Right leg flaccid. A lumbar puncture yielded 2.5 cc. of fluid containing excess of white corpuscles. Nov. 13. The condition was unchanged; the animal was etherized. The organs generally were normal in appearance; the spinal cord was edematous. Microscopic examination of sections of the spinal cord, medulla, and interstitial ganglia revealed the characteristic lesions of poliomyelitis. The blood vessels and ground substance showed infiltrations with mononuclear cells; the motor nerve cells were degenerated and invaded by phagocytes.

Dec. 3. An emulsion of the glycerolated spinal cord and medulla was injected into each sciatic nerve and the peritoneal cavity of a *Macacus cynomolgus* (Monkey B) and a *Macacus rhesus* (Monkey C). Dec. 9. The *rhesus* monkey was noted to be excited. Dec. 10. Lumbar puncture yielded 3 cc. of turbid fluid containing excess of white cells. By Dec. 13, the legs were partially paralyzed; the animal was etherized. Microscopic sections of the spinal cord, medulla, and intervertebral ganglia showed typical infiltrative and degenerative lesions of poliomyelitis. The *cynomolgus* monkey became excited on Dec. 10, and on the 19th paralysis of the legs appeared. By Dec. 21 the arms and back were weak, and the paralysis was extending. Dec. 23. The animal was etherized. The general viscera appeared normal, but the spinal cord was both edematous and congested. The microscopic sections of the cord, medulla, and intervertebral ganglia showed typical infiltrative and degenerative lesions attended by neurophagocytosis. Subsequently the glycerolated specimens of the nervous organs of Monkeys B and C were used for inoculating still other monkeys, in which typical paralysis was induced.

The conclusion drawn by the authors from this demonstrative experiment was to the effect that the parents of E. A., neither of whom showed any symptoms of illness and who evidently were not suffering from poliomyelitis, harbored the virus of the disease in the nasopharynx. Hence the existence of the healthy carrier was thus established experimentally.

The next demonstrative experiment was supplied by Kling and Pettersson⁶ who, in referring to their earlier failure to produce clinically and anatomically typical poliomyelitis with nasopharyngeal washings, attribute the failure to the injection of insufficient amounts of virus into the monkeys. They repeated the tests, using washings concentrated *in vacuo* with the Faust-Heim apparatus.

They started out by determining the heat lability of the active virus, and ascertained that a liter of fluid carrying an effective dose could be evaporated at

temperatures ranging from 35 to 38°C. to 200 cc. without losing its potency. They now obtained nasopharyngeal washings in amounts of 1 to 2 liters from healthy persons in contact with cases of acute poliomyelitis. In one instance in which the washings were taken from the healthy members of a family in which one member had recently died of acute poliomyelitis, the inoculation resulted successfully.

The patient was a male, age 41 years. The illness began on Sept. 10, the legs becoming paralyzed 2 days later. Death took place on the 4th day of illness from respiratory failure. The surviving members of the family consisted of the wife and three children ranging from 10 to 14 years, all remaining well. One day after the death of the father in a hospital, nasal washings were taken in distilled water from the surviving members of the family. The combined washings, amounting to 1 liter, were evaporated *in vacuo* to 75 cc., sodium chloride was added, and the mixture was filtered first through paper and then through a Berkefeld candle.

Sept. 20. 0.5 cc. of the filtrate was injected intracerebrally and 20 cc. were introduced into the peritoneal cavity of a *Macacus sinicus*. Oct. 2. The right leg and on the next day both legs and back were paralyzed, and death resulted. The microscopic sections of the spinal cord showed moderate perivascular and diffuse infiltration of the nervous tissue with mononuclear cells and neurophagocytosis. Oct. 3. A second *Macacus sinicus* was inoculated intracerebrally and intraperitoneally with an emulsion of the spinal cord of the first animal. On Oct. 13 the right leg and on the next day the left leg were paralyzed. Oct. 15. The animal was killed. Sections of the spinal cord showed typical infiltrative and degenerative lesions of poliomyelitis.

There can be no doubt, therefore, that in this family one or more healthy carriers of the active virus of poliomyelitis existed. That the result was not due entirely to the employment of concentrated washings is indicated by the failure to detect the virus in the washings obtained from the healthy associates of two other cases of acute poliomyelitis.

OBSERVATIONS.

In the two successful instances just reviewed, mixed washings were employed for inoculation. It is, therefore, impossible to state whether one or more of the healthy contacts of the cases of poliomyelitis were carriers. In the instance which we shall report the individuals were irrigated separately. The final result proved that more than one virus carrier was present, and it was demonstrated that such a healthy carrier may develop poliomyelitis. We may

therefore regard the chain of the mode of infection as now having been completed for the first time. The separate links may be defined as follows:

Case of acute poliomyelitis → contact carrier → second case.

A still further analysis would determine that through the contact carrier other carriers occur, among which a certain number of additional cases arise.

Poliomyelitis occurred in epidemic form in Washington County, Vermont, in the summer of 1917. From June 1 until September 1, 79 cases were recognized among the population of 45,000.

Carey P., male, age 16 years. The patient lived in the village of Waitsfield, 18 miles from Montpelier, where cases of poliomyelitis existed. No case of the disease had been discovered in Waitsfield. On June 2, 1917, he attended a ball game at Northfield where there were no cases, and returning home stopped in Montpelier for supper. Probably in the assembly at Northfield persons from the infected district were present. Until June 12 there were no symptoms of illness; on that day there was complaint of headache and pain in the back and legs. The patient vomited once. June 13. First seen by a physician who observed that the patient had fever, and treated him for a gastrointestinal upset. June 16. Extensive paralysis involving both legs, right triceps, intercostals, pectorals, and diaphragm. Lumbar puncture yielded clear fluid under pressure, containing 400 white cells per cmm. and excess of globulin. Death occurred on this date.

The family consisted of the father, age 59 years, mother, age 42, sister, Hazel, age 13, two brothers, Everett, age 10, and Dwight, age 7. The two younger brothers slept in the same bed, and in the same room with the elder brother Carey.

June 16. Everett and Hazel were given nasopharyngeal irrigation with distilled water, 60 cc. being obtained from the former and 100 cc. from the latter. 10 per cent of ether was added to each, and the fluids were sent at once to the laboratory. One of us had previously determined that ether inhibits bacterial development without injuring the poliomyelitic virus. The washings were treated separately as follows: Glass beads were added and they were shaken mechanically for $2\frac{1}{2}$ hours. They were then centrifuged at high speed for $2\frac{1}{2}$ minutes, and the supernatant fluid was passed through a Berkefeld N candle and concentrated *in vacuo* by the method already described by us⁸ at 35°C. to 2 cc. The entire concentrate was injected intra-

⁸ Amoss, H. L., and Taylor, E., *J. Exp. Med.*, 1917, xxv, 507.

cerebrally into two *Macacus rhesus* monkeys (Monkey A (Everett) and Monkey B (Hazel)). The time elapsing between the collection and the injection of the washings was less than 6 hours.

We return briefly to the history of the two children. Everett had not been away from the village and was in usual health until June 13, the day after Carey fell ill. He also felt indisposed, showed a temperature of 102° F. and suffered from diarrhea, but did not vomit. However, he recovered quickly and subsequently on minute examination has shown no muscular weakness or abnormality of reflexes.

Hazel had not been away from Waitsfield. She had been entirely well at the time the washings were taken and remained well until June 21, at which time she complained of headache. She showed a temperature of 102° F. On June 22 her reflexes were exaggerated and stiffness of the back was present, but no muscular weakness was detected. Lumbar puncture was unsuccessful. The symptoms subsided gradually, but reexamination made on July 22 revealed partial paralysis of the left deltoid, right anterior tibial, and abdominal muscles. She had, therefore, suffered a mild attack of poliomyelitis.

Monkey A.—June 16, 1917. Inoculated. Remained well until June 29, when it was excitable, emitted staccato cries, and showed ruffled hair. The animal was noted to be clumsy in movement and unable to jump. June 30. Both legs were weak. July 4. The right leg was paralyzed and flaccid; the left leg and back were weak. The paralysis of the left leg and back, but not of the right leg, disappeared; the latter remained and contracture gradually set in. At the present time (Sept. 1) the contraction of the right leg is so marked that in moving about the animal does not touch the limb to the floor. On Aug. 8 blood was withdrawn for a neutralization test and at the same time an intracerebral inoculation was made with a large dose of virus proved active in another monkey; the result was negative. The animal, as is usually the case, having recovered from a recent infection, was resistant to reinoculation.

Monkey B.—June 17, 1917, 3 a.m. Injected intracerebrally with 1.5 cc. of the concentrated washings. Recovery from the anesthesia was immediate, and the first symptoms, consisting of excitability, ruffled hair, staccato cries, and partial paralysis of the right leg, were observed. June 26. The paralysis being stationary, the animal was etherized. The organs appeared normal to the naked eye. Microscopic sections revealed, however, marked typical lesions of poliomyelitis. They affected the spinal cord (Fig. 1), medulla (Fig. 2), and intervertebral ganglia (Figs. 3 and 4), and consisted of typical infiltration with mononuclear cells and nerve cell degeneration with phagocytosis.

Monkey C.—June 26, 1917. Injected intracerebrally under ether anesthesia with 2.5 cc. of a 20 per cent emulsion of spinal cord and medulla of Monkey B. July 7. The first symptoms were noted, consisting of ruffled hair and inclination of head to the left. July 8. The animal was ataxic and protected the right leg. July 9. Unable to jump; legs and back weak. July 10. Paralysis progressing. July 15. Etherized. The spinal cord showed typical focal lesions of poliomyelitis in which cicatrization was beginning.

These experiments leave no doubt that the washings, both from Everett and from Hazel, contained the virus of poliomyelitis. The instance of Hazel is of particular importance since in her case the virus was detected in washings taken 5 days before the first symptoms of what proved subsequently to be a mild attack of poliomyelitis set in. In other words, she was carrying the virus in her nasopharynx several days in advance of the appearance of any signs of illness. She constitutes, therefore, an example of a carrier of the virus developing poliomyelitis—the first one in which the demonstration has been proved experimentally.

The interpretation in the case of Everett is not so simple. When the virus was detected in his nasopharynx he had passed through a slight attack of illness, at about the same time with, and of about the same character as that of his brother Carey who died, but unattended by paralysis. The presumption is that Everett suffered from a non-paralytic or abortive attack of poliomyelitis. The detection of the virus in his case proves him not to have been a healthy, but a recovered carrier of the microbic cause of the disease.

The two children having been shown to be virus carriers, their nasopharyngeal secretions were tested by the method of Amoss and Taylor,⁸ to determine whether they would neutralize an active poliomyelitic virus.

July 23, 1917. Washings with sterile water were taken from the children, and fractionally sterilized and mixed. To 15 cc. of the mixture were added 3.75 cc. of a Berkefeld filtrate of a 5 per cent stock glycerolated poliomyelitic spinal cord. After shaking, the combined fluids were permitted to remain at 37°C. for 24 hours. 1 cc. of the fluid was injected intracerebrally into a *Macacus rhesus*. No symptoms appeared until Aug. 4, when excitability, ataxia, paralysis of the right arm, and weakness of the back were noted. Aug. 8. Animal prostrate. Aug. 10. Died. The microscopic lesions were typical of poliomyelitis.

The mixed nasal washings failed, in this experiment, to neutralize the virus.

The youngest child, Dwight, age 7 years, was refractory and no washings were obtained from him on June 16 when they were taken from the other children. On June 18 he complained of being unwell. The symptoms were severe headache, stiffness of neck, exaggerated reflexes, but no diarrhea. Lumbar puncture yielded a fluid containing 500 white cells per cmm. and an excess of globulin. Immune poliomyelitic serum from recovered cases of the disease was administered intraspinally, intravenously, and subcutaneously: 24 cc. were given intraspinally, 30 cc. intravenously, and 39 cc. subcutaneously. Recovery was prompt, with a slight paralysis of the right anterior tibial muscle. Nasopharyngeal washings were, however, obtained on September 4, which after filtration and concentration were inoculated into a *Macacus rhesus* (Monkey D). The monkey remained well.

DISCUSSION.

This series of cases of poliomyelitis in one family, with the circumstances surrounding their origin, forms an instructive illustration of the mode of infection of the disease as brought out by the clinical and experimental study.

In the first place, one child only—the eldest boy, Carey—was exposed in a locality in which poliomyelitis was epidemic. The exposure took place on June 2. Immediately afterwards he returned home, to a village in which no previous case of the disease had occurred, and mingled freely with his younger brothers and sister. The contacts may be considered to have been intimate in that the three male children slept in the same room, two of them in the same bed.

The incubation period in Carey's case was 9 or 10 days, as he was taken ill on June 12. His brother Everett, 6 years younger, developed symptoms 1 day later and passed through what was probably a non-paralytic attack of poliomyelitis. He may be considered as having been infected by Carey some time during the incubation period, and to have exhibited a shorter incubation than his brother. The youngest brother, Dwight, was also freely exposed to both older brothers and exhibited symptoms passing into those indicative of

poliomyelitis 5 or 6 days later than his brothers. Finally, Hazel, the sister, in age between the two older brothers and possibly less freely exposed, developed symptoms and muscular weakness last of all and about 10 days after the eldest brother. The incubation periods of the cases, therefore, probably were 10 days or less, and the order of the attacks was such as to indicate successive infection and not a common one.

The second feature worthy of emphasis is the detection in this one family of two carriers of the poliomyelitic virus by the inoculation test. One (Everett) was discovered to be a carrier probably following a non-paralytic attack. In the instance of Hazel there is no doubt, first that she was discovered to be a healthy carrier, and second that she developed typical poliomyelitis during the period of carriage. Incidentally the nasopharyngeal secretions of Hazel and Everett failed to neutralize the poliomyelitic virus.

If the view that the mode of infection in epidemic poliomyelitis is by way of the nasopharyngeal mucous membrane and is brought about or greatly facilitated through the operation of healthy carriers of the virus is accepted, we may well consider whether in the final analysis every case of the disease does not develop from a carrier. At first this may seem startling, and yet it merely means that after contamination of the nasopharynx with the virus, an intervening period exists during which persistence, multiplication, and invasion of the virus take place. In not all contaminated persons does this process become complete; in some the virus may merely persist for a time, in others it may multiply in the nasopharynx (these constitute the healthy carriers of greater or less endurance), while in the exceptional few invasion also occurs. In the last class symptoms arise, and these individuals become cases of poliomyelitis.

SUMMARY.

A family group containing four children of whom all showed in varying degree symptoms of poliomyelitis is described. The source of infection and periods of incubation have been followed. Two of the children were proven by inoculation tests to carry the virus of poliomyelitis in the nasopharynx. Of these, one was detected to be

a carrier after recovering from a non-paralytic attack of the disease, and the other was discovered to be a carrier about 5 days before the initial symptoms, attended later by paralysis, appeared. The original case from which the three others took origin was fatal; the youngest child, after quite a severe onset, was treated with immune serum, and made a prompt and almost perfect recovery. The nasopharyngeal secretions of two of the cases, taken 1 month after the attack, proved incapable of neutralizing an active poliomyelitic virus.

The proposition is presented that every case of poliomyelitis develops from a carrier of the microbic cause, or virus, of poliomyelitis.

EXPLANATION OF PLATES.

PLATE 55.

FIG. 1. Spinal cord of Monkey B. showing perivascular infiltration and neurophagocytosis. $\times 90$.

FIG. 2. Medulla of Monkey B, showing diffuse mononuclear infiltration, nerve cell degeneration, and neurophagocytosis. $\times 230$.

PLATE 56.

FIG. 3. Intervertebral ganglion of Monkey B showing infiltrative changes and nerve cell invasion. $\times 120$.

FIG. 4. Intervertebral ganglion of Monkey B, showing mononuclear infiltration, nerve cell degeneration, and neurophagocytosis. $\times 240$

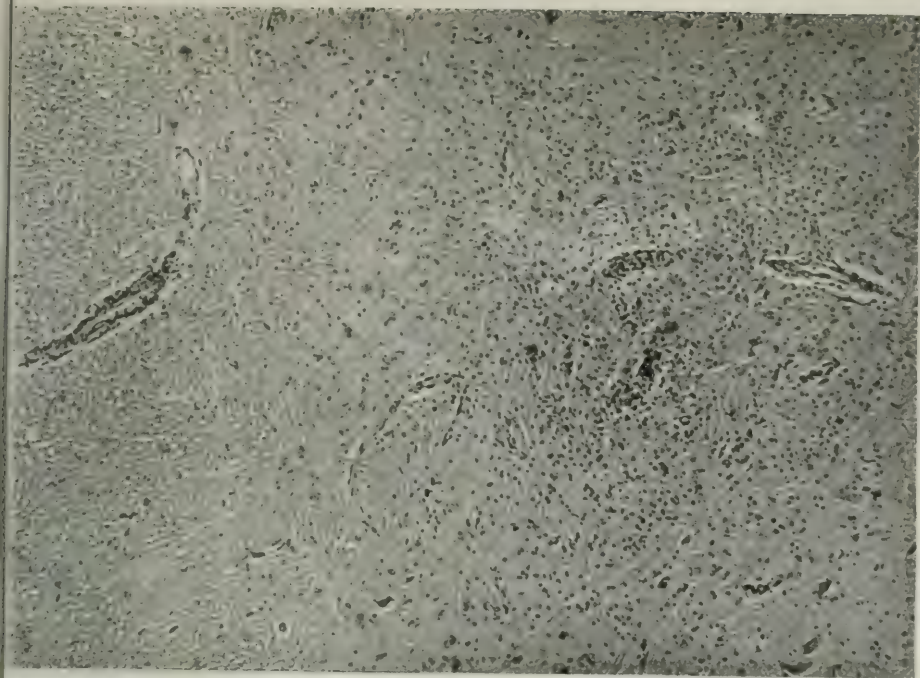


FIG. 1.

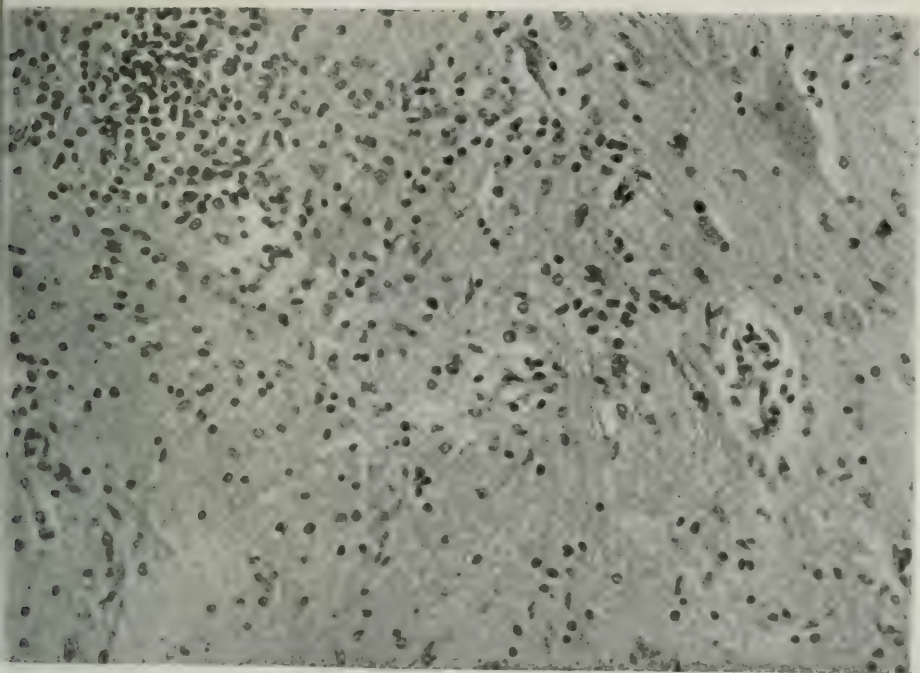
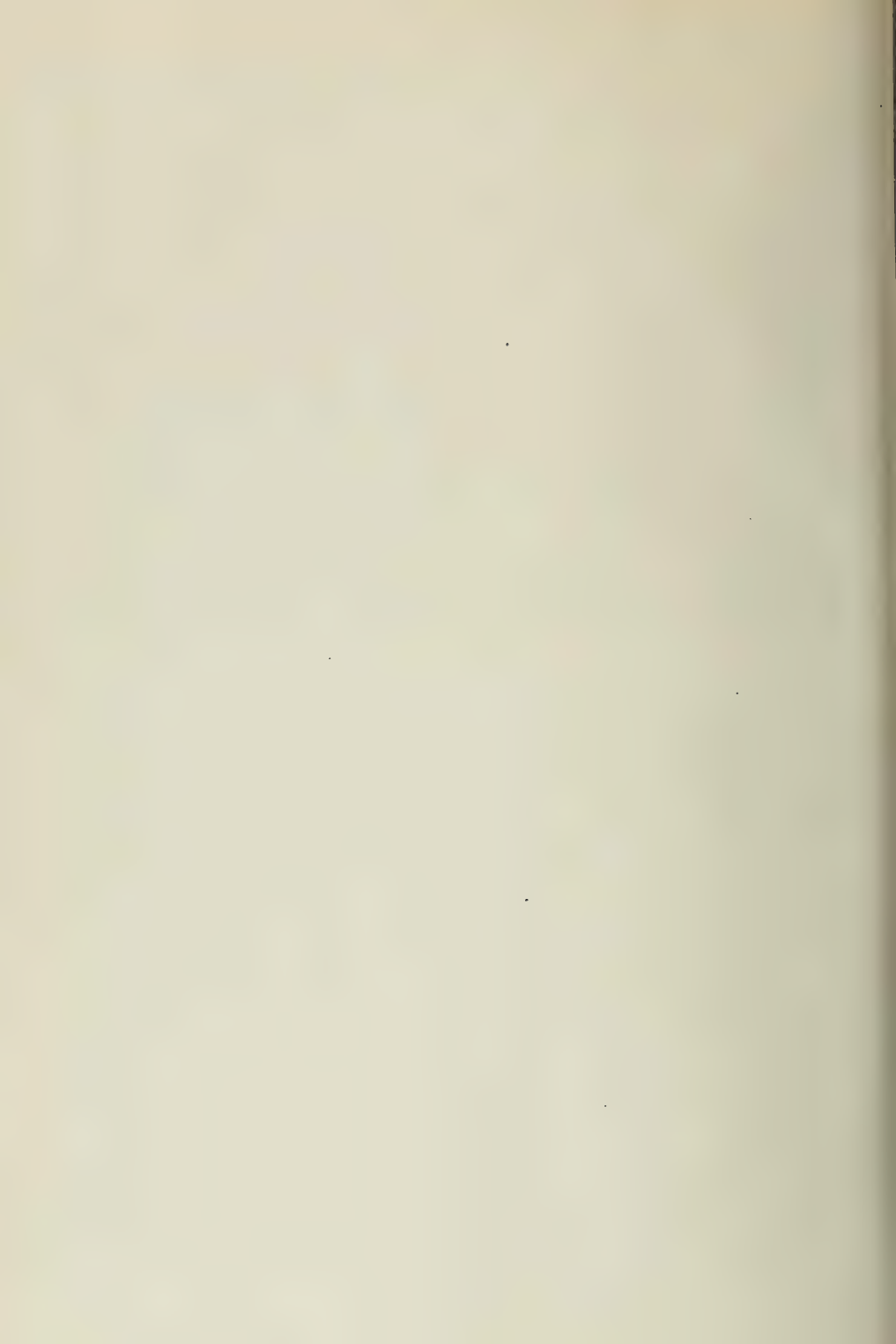


FIG. 2.

(Taylor and Amoss: Carriage of the virus of poliomyelitis.)



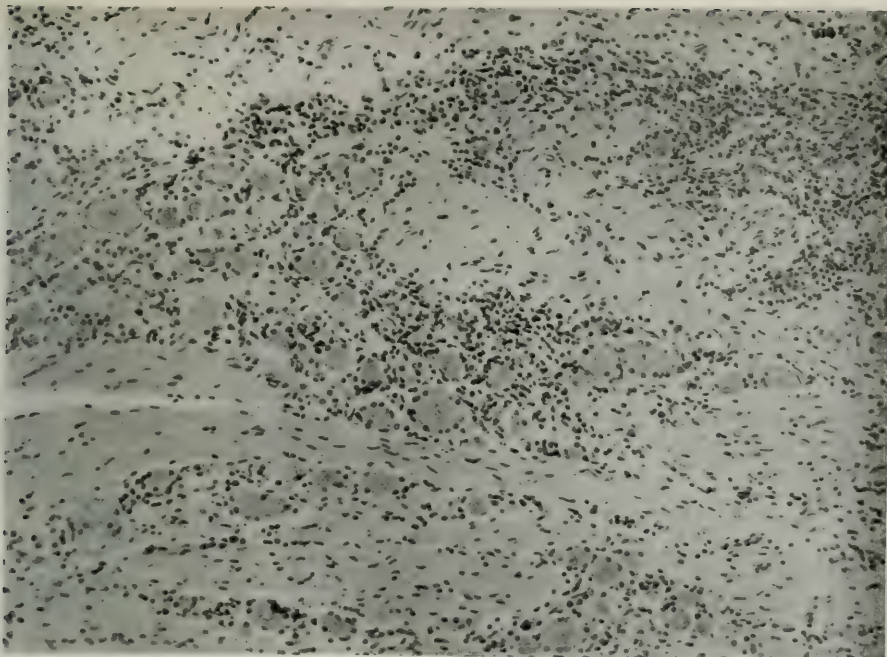


FIG. 3.

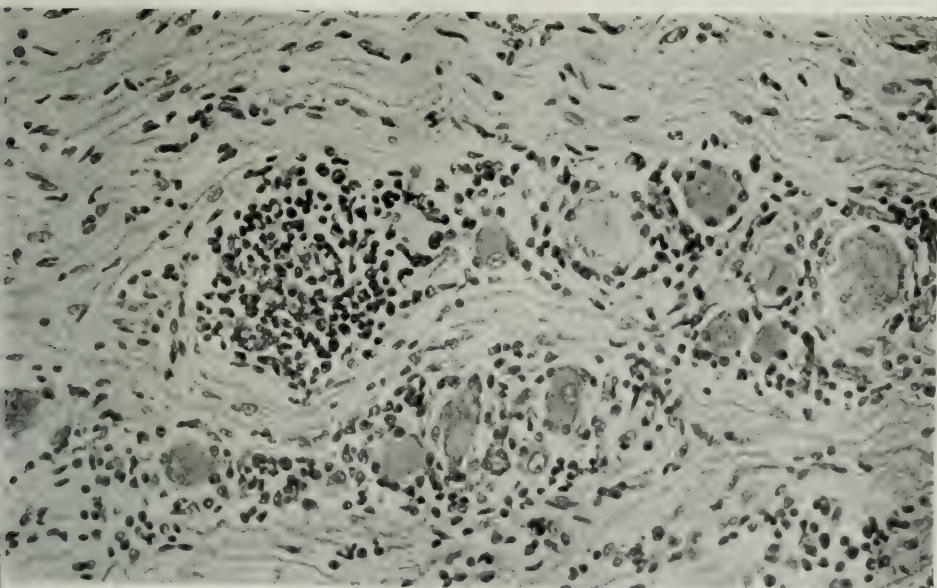


FIG. 4.

(Taylor and Amoss: Carriage of the virus of poliomyelitis.)



A FURTHER CONTRIBUTION TO THE METAMORPHOSIS OF AMPHIBIAN ORGANS.

THE METAMORPHOSIS OF GRAFTED SKIN AND EYES OF AMBLYSTOMA PUNCTATUM.

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FIVE PLATES AND THREE TEXT FIGURES

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I. INTRODUCTION.

In a series of experiments performed on larvae of *Salamandra maculosa* in 1910, 1911 and 1912,¹ the writer studied certain factors involved in the normal metamorphosis of the Amphibian eye. It was shown that eyes taken from an old larva and grafted to a young one, cannot metamorphose unless the host metamorphoses. The metamorphosis of the old eyes, therefore, was delayed as compared with the time of metamorphosis of their eye-mates left on the old larvae from which the grafted eyes were taken. In one case in which the new host metamorphosed two months later than the animal from which the grafted eye was taken, the metamorphosis of the grafted eye also occurred two months later than that of its eye-mate. Furthermore, it appeared that the grafted eye must metamorphose at the time that the eyes of the host metamorphose. When one eye of a young larva was grafted to an old larva, the metamorphosis of the young eye was accelerated just so much as to occur simultaneously with that of the host's eyes. In this way it was possible to accelerate metamorphosis of the grafted eye by two to six months. From these experiments, the following conclusion seemed justified: that metamorphosis of the eye is dependent upon the presence of a particular factor without which metamorphosis cannot occur, and that this factor cannot be produced by the eye itself, but is developed in another part of the animal's body whence it is furnished to the eye.

When however, very old larvae such as were leaving the water one or more days after the operation, were one of the two components (host or graft) in these grafting experiments, the results differed considerably from those described above. When one of these very old larvae (called 'Larvenendstadium' in the first publication) was the host the young grafted eye metamorphosed at a later date than the host and furthermore, when such an old eye was grafted on a young larva it metamorphosed long before and independently from the host. Though in these 'Larvenendstadien' the morphological change corresponding to metamorphosis was not developed at the time of operation, the factor necessary to its production was apparently already contained in the eye. The young eyes grafted to the

¹ E. Uhlenhuth, 1913 a.

old animals on the other hand, did not metamorphose simultaneously with the host, because one or two days of action of this factor is apparently not sufficient to produce metamorphosis.

Thus, in the case of the eye of *Salamandra maculosa* the existence of a definite factor involved in the metamorphosis was shown. It was found (1) that this factor cannot be produced by the eye itself but originates in the body of the host, the exact part being unknown; (2) that it must act a certain length of time in order to start the process of metamorphosis and (3) that metamorphosis will occur if once induced by this factor, even if the organ is removed from the further influence of the action of the factor.

The fourth characteristic of the metamorphosis factor could not be precisely determined as the number of experiments was not sufficiently large. It was observed that eyes of salamander larvae also metamorphosed when grafted to larvae of Triton. These experiments suggested that the metamorphosis factor is a non-specific agent, but owing to the small number of experiments no comment was made at that time.

The first three characteristics however, were observed with such regularity that they were considered to be definite rules of the process of metamorphosis and apt to throw some important light on this phenomenon. In order to justify this opinion it was considered particularly necessary to examine several of these organs, the metamorphosis of which is most prominent and to find out whether the metamorphosis of these organs is subjected to similar or identical rules as the metamorphosis of the eye. If this were so, one would be justified in thinking that one single factor governs the metamorphosis of all these organs and that a common center in the body responsible for the development of this metamorphosis factor could be found.

It was from this idea that Kornfeld's experiments on the metamorphosis of the gills originated. By grafting the gills in a similar way as the eyes, it was proved that also in the metamorphosis of the gills a metamorphosis factor is involved, which possesses the first characteristic of the factor governing eye-metamorphosis. No experiments were made to examine the three other factors.

In 1913, several months after the author's first publication on the subject in question, Weigl's paper on homoplastic and heteroplastic

transplantation of the Amphibian skin appeared. Some of his experiments were made in exactly the same manner as the author's eye-grafting experiments. To the facts obtained by these experiments we will have to refer later on. The conclusions however, which Weigl pointed out in a chapter on metamorphosis, were entirely contradictory to our results. From his experiments Weigl formed the opinion that the metamorphosis of the skin is a process of absolute self-differentiation of this organ (Roux)² while we found on the contrary, that the eye is unable to metamorphose unless it is furnished by the animal's body with a certain factor or agent, which alone can induce metamorphosis of the organ. If the conclusions of Weigl were right, the theory that metamorphosis of the organs is enacted from a common center in the body would certainly lose much of its interest and probability.

Meanwhile however, Gudernatsch's experiments on feeding tadpoles on thyroid, the first results of which were reported only a short time before the writer had finished his first group of experiments, have been repeated by several authors (Romeis, Kahn and others) and it is certain that thyroid substance can induce metamorphosis of the whole animal within a relatively short time. This again suggests that in normal metamorphosis the development of all organs, which take part in it, is governed by one center and that metamorphosis of these organs is not a process of self-differentiation.

Furthermore J. Loeb, following the ideas of Sachs, in a recently published book, has developed a theory according to which the flow of certain substances plays a very important rôle in the development of the organs. A large number of facts are quoted which show that the mode of distribution by this flow of the substances determines the development of a certain structure at a certain place.

And finally, certain experiments are in progress and have been partly reported (Adler, Smith, Allen, Hoskins) by means of which one single organ (Hypophysis, Thyroid) has been removed from the Amphibian body, and it seems that the absence of this single organ actually prevents or modifies metamorphosis in some way.

From all this it seems that a study of the process of metamorphosis of the skin gains renewed interest and that the demonstration of a

² R. Weigl, p. 620.

metamorphosis factor acting on the skin in a way similar to that of the metamorphosis factor involved in the metamorphosis of the eye and the gills, would be of great importance. For this reason a series of grafting experiments on *Amblystoma punctatum* and *Amblystoma tigrinum* were performed in the late spring of 1916. Special attention was paid to the metamorphosis of the grafted skin but in several cases the metamorphosis of the eye was also watched.

II. Certain Morphological Characteristics of the Metamorphosis of the Normal Skin and Eye in Amblystoma punctatum.

A. Skin.

As can be seen from the foregoing pages and will better be seen from the next chapter, the method of these experiments is such as to compare the skin of different individuals as to the time at which metamorphosis occurs or as to the rate at which development progresses. This cannot be done unless we have certain definite characteristics at hand which mark the time of the entrance of the skin into each stage concerned in this comparison. Therefore it is necessary to outline here briefly the course of the development of those characteristics which we have chosen for the purpose of indicating the entrance into certain developmental stages and to signify what characteristics we have decided to be our indicators.

It is well known that during metamorphosis the Amphibian skin is subjected to a large number of changes, some of which concern only the histological structure, while others are of such a nature as to alter the external aspect of the organ also. It is clear that we could not use the first ones, as the nature of our experiments does not permit of killing the animals for examination. Among the external changes only the development of the permanent coloration of the skin was used. As we shall see it is possible to discern a number of definite stages within the process of working out of the skin colors. The development of each of these stages may, and according to what has been said on page 32 of this paper actually does correspond to certain more or less complicated internal physiological processes, which are visible neither to the microscope nor to the naked eye; by

means of these processes a number of internal changes may be effected, the final expression of which are those morphological changes with which alone we will be concerned in this paper. The nature of the internal changes connected intimately with the production of the pigments may be important and so far as the gathering of a certain kind of chromatophores at certain places into single spots is concerned seems to be of particular interest, but need not be discussed here. Concerning the action of the physiological processes, certain features of their nature have been disclosed by experiments similar to those to be reported and these are described in former papers concerned with these experiments.

Before entering into a description of the development of the skin coloration it should be mentioned that among the American species of Salamanders available for our experiments, there is none in which the development of the skin shows such a definite step as is the appearance of the yellow spots in the European species *Salamandra maculosa*. In *Amblystoma punctatum* the development of the permanent skin patterns of the adult is much more continuous and gradual; nevertheless as we will see it is possible to divide the development of the yellow spots of the adult animal into a number of well defined developmental stages.

The first color stage of the skin prevails during the largest part of the larval period; it is characterized by a more or less uniform pigmentation of the entire skin except the skin of the belly, which however does not enter into our experiments. This uniformity is produced during the first days of life by a continuous layer of a yellowish pigment which is not contained in chromatophores, but exhibits a purely diffuse character. Soon after the animals are hatched, melanophores appear scattered more or less evenly throughout the skin of the head, back and tail. With the gradual increase of the number of melanophores the skin continually darkens without losing the uniformity of its color. No definite patterns are worked out on the skin during this period, which shall be referred to as 'even yellow brown' in the following chapters. Of course, there are many variations to be observed corresponding with individual differences as well as with differences in the treatment of the animals and age of the animals; but none are definite enough to play any rôle here. Varia-

tions from a very light to a very dark shade usually correspond with progressing age and also with similar differences of the color of the background on which the animals are kept, with the intensity of light and the differences in temperature. Grayish, reddish or greenish tints may be intermingled but without changing the uniformity of the coloration. Figure 1 indicates this stage; it shows a larva of *Amblystoma punctatum* about six months old, kept in a dark room at 15°C. and fed on worms. The same stage may be seen from figure 13.

The next stage is the 'network.' It is characterized by the appearance of a yellowish or greenish network on an even yellow brown background. It also shows great variations: it may be very dense or very loose; it may be produced by either fine and numerous yellow streaks or by fewer and broader stripes and blots. It may be of intense yellow or of a greenish color. Similar factors to those mentioned in the last paragraph seem to produce these variations and we shall deal with some of them in another paper. Here it is important to mention that the network can be sharply defined as soon as it appears, though considerable time is required to develop all of its characteristics definitely. It always develops while the animal is still in the larval period. Figure 2 pictures the network as it appeared on a larva six months old, kept in a dark room at an average temperature of about 15°C. and fed on worms.

The next stage may begin while the animal is still in the water and under these circumstances indicates that the animal will soon leave the water, or it may begin after the animal has left the water. At any rate, it always begins about this period, and the time of its appearance and further development corresponds to the period in which the animal as a whole undergoes those processes which are generally called metamorphosis in the Amphibians. (See Chapter III, p. 44.) For this reason this stage is considered here as corresponding with the metamorphosis of the skin. The beginning of this stage is indicated by a general contraction of the network, by means of which it loses its anastomoses and appears in the form of isolated spots or blots, a phenomenon which is—within the limit of a few days—well defined in respect to time. But the development of the definite yellow spots as they appear on the adult animal, occurs very gradually

in most animals requiring about seventeen to twenty-one days and in some exceptional cases much more. After the animal has left the water, all colors soon appear quite dim in consequence of the development of relatively dry and dull layers of horny cells lying on the surface of the skin and unshed for a long time. During this period, the spots decrease in size and change their shape, assuming a round form in the majority of animals. Many spots disappear. This stage was called "Separation of the network." Unfortunately no photograph was taken of a worm-fed larva entering this stage. In its place a photograph of a thymus-fed animal kept at a temperature of about 25°C. is shown here (fig. 3); it was taken a short time after the separation of the network had started. Although there are certain differences between the color of thymus-fed and worm-fed animals, the stages of development show the same essential features in both cases. The day before this animal was photographed, 102 yellow spots were counted on the right side of the animal, two and one-half months later, when the animal was fully metamorphosed, only 32 spots were left.

Figure 4 shows the separation of the network at a later period on a worm-fed animal which left the water eleven days before it was photographed and painted. This picture also shows that the development of the spots on the tail has progressed more rapidly than on the body. We shall refer only to the spots on the body and head.

The last stage which we call 'black' is reached when the colors of the spots and background have fully cleared up. It corresponds with the termination of metamorphosis. The background then appears black and the spots are of a varying bright yellow; the colors also are more vivid because the surface of the skin is now glossy instead of dull. The transition from the third into the last stage occurs very gradually and no definite line can be drawn between them. Figure 14 shows a fully metamorphosed animal.

In the following text the terms 'cinnamon' and 'brown' refer to the color of the background after the animal has left the water. In the beginning it very often assumes a reddish brown color which is called 'cinnamon'; then this color changes into a greenish black-brown which is called 'brown.' By getting gradually darker and darker this color becomes black.

It should also be mentioned that the rest of the skin in which the network and the spots seem to be imbedded, is called "Background."

Finally it must be said that size and shade of the yellow spots vary greatly as we shall see later on, when we shall deal with this fact. One exception should be mentioned here. A very small percentage among a certain number of animals does not develop any spots at all on the back, or only very few of small size and of a faint greenish color. They seem to consist of a number of tiny yellowish pigment dots, which attach a loose aspect to them; they are called 'dotted spots.' In such animals the rate of development of the background is more rapid than the development of the yellow spots; at a similar stage of development of the background in other animals the spots would have been better developed than in the animals referred to.

The four stages which we have found during the development of the skin are (1) even yellow brown, (2) network, (3) separation of network and (4) black; the third one, because of its simultaneity with the general metamorphosis of the whole animal, has been considered to directly correspond with the metamorphosis of the skin. But since the rate of development of the other stages and particularly also of the second stage has been found subjected to the same laws as that of the third stage, there will be no need of emphasizing this artificial distinction when we discuss the general laws to which the development of the skin appears to be subjected. The second and the third stage together constitute the phenomenon of the working out of one typical characteristic of the adult, i.e., the yellow spots, which is the first step of this stage.

B. Eye.

In the eye also certain changes of its pigmentation were used as a mark of the entrance into metamorphosis.

In the beginning of the larval period the eye shows, when examined with the naked eye, a continuous rather light yellow ring, which corresponds with the iris and contains no black pigment. The color of this ring darkens with increasing age but no black pigment forming interruptions between the yellow parts of the ring are visible to the naked eye. Some time before the larvae leave the water the ring becomes narrower and finally forms a distinct and narrow but still con-

tinuous line around the pupil. Several days after the animal has left the water, the ring undergoes a distinct and very sudden change; the yellow line breaks up into numerous yellow and black dots. After this there occurs a very gradual change, which stretches over a long period; while the black dots become larger, the number of yellow dots decreases until finally pupil and iris form one black circle on the outer margin of which a small number of yellow dots are scattered. In some individuals no dots remain while in others yellow dots are present for several months. Such yellow dots may be detected occasionally even in adult individuals.

On examination with a high power binocular lupe the phenomenon described above appears somewhat different. From the beginning a small number of contracted melanophores can be seen scattered throughout the yellow iris. The darkening of the iris is not due so much to an accumulation of melanophores the number of which increases very little, but to the appearance of orange or reddish yellow blots. In these blots the blood can be seen circulating while in the early stage the blood circulation could not be seen. It also seems that some of the yellow chromatophores themselves have become somewhat darker yellow. In the very early stages a division into two sections can be seen—an inner narrower section and an outer wider section. The inner section does not contain any melanophores at all and appears even under a weak magnification of the microscope as a continuous ring of pure yellow pigment (fig. 5). This division into two sections later on becomes more accentuated as the reddish yellow blots only appear in the outer section. Finally, in the outer section the amount of darker pigment increases and large parts of it appear brown while the inner section still remains without melanophores. It is at this time that to the naked eye the ring seems to have become narrower since the outer section has become black to the naked eye. At the time that the naked eye sees the breaking up into black and yellow dots, the yellow chromatophores of the inner section have started to loosen up and form—by means of their anastomosing processes—a fine network through the meshes of which the black epithelial layer of the iris seems to become visible. Soon the yellow chromatophores become fewer and cease to anastomose with each other. This process and a gradual decrease in the

number of isolated yellow chromatophores correspond to the decrease in the number of yellow dots seen by the naked eye; each yellow dot corresponds to one single xanthophore (fig. 6).

The stage of breaking up into yellow and black dots will be assumed to be the beginning of the eye's metamorphosis.

III. The Experiments.

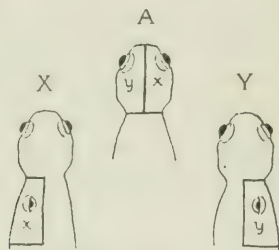
A. Homoplastic Transplantation Between Larvae of Amblystoma punctatum.

1. Material and Method.

The general method of these experiments should be to remove one piece of skin from the larva just before the factor inducing metamorphosis is developing in the body, and to graft the piece to another larva in which this factor will not develop for a long time. The animal from which the skin graft is taken should metamorphose long before the piece of skin which was grafted onto the young host would metamorphose; provided the metamorphosis of the skin follows the same rules as that of the eye and gills. Grafting of a young skin to an old larva in which the metamorphosis factor is just to develop should give a result opposite to that of the first mentioned experiment; metamorphosis of the skin graft should be accelerated and should occur before the animal from which the graft was taken would metamorphose.

In order to use this method, it is necessary to have on hand larvae whose age varies sufficiently to make it certain that with a younger animal also, an earlier stage of development will correspond, which relation according to a well known experience exists in Amphibians, only if the individuals differ in age by months. Since we used larvae whose age varied only by a few days we had to modify our method. The new method was based on the fact that in a lot of animals hatched on the same date, the rate of development varies, sometimes to the extent of several months, in spite of apparently the same treatment. It was hoped then, that of two individuals chosen at random, one might develop more rapidly than the other; by increasing the number of pairs there would also be an increase in such pairs whose individuals would develop unequally. Thus each experiment consisted of two

animals, X and Y (text fig. a), to each of them one of the two eyes of a third animal A and the skin of the corresponding half of the head of animal A was grafted. If left with animal A the skin of both halves of the head would have metamorphosed at the same time; namely: at the time when the factors necessary for the metamorphosis of the skin after they have developed in the body of animal A, and have been carried into the skin, have acted in the skin a sufficient length of time to cause the physiological changes necessary to produce the morphological changes of the skin described in the preceding chapter. But removed from the animal A and separated from each other—one half having been grafted to X, the other half to animal Y—they would not metamorphose simultaneously with each other but simultaneously with their respective new hosts and hence at dif-



TEXT FIGURE a.

ferent times; provided of course, that metamorphosis of the skin followed the principles observed in the metamorphosis of the Amphibian eye and gills.

To use the skin of the head for grafting offers a special advantage. As pointed out in Chapter II, in studying metamorphosis of the skin of *Amblystoma punctatum* we must choose some definite characteristic of the skin. For certain reasons explained in Chapter II the appearance of the yellow spots was found to be the most convenient time-mark. In the larvae, however, where of course no spots are developed yet, it is impossible to foresee on what parts of the skin of the body such spots will develop and as the distribution of the yellow spots is subjected to great variation there would be little chance of having selected for grafting a piece of skin that would finally develop a spot. But this is different for the head, as in the majority of animals one or two yellow spots will develop on the skin

above both sides of the lateral 'os occipitale.' Among 83 individuals for instance, which were examined in this respect, only 16.9 per cent did not have any spot on the right side (only this side was counted) of the caudal end of the head, while 83.1 per cent of the animals had developed one or more spots. Therefore, if the skin of the head is used, the chance of obtaining yellow spots in the graft is about 83 per cent and one is also prepared at the time of metamorphosis to look for these spots on the rear end of the skin graft, provided that the skin be grafted in such a way that its front and rear correspond to cranial and caudal respectively of the new host. In the experiments the actual number of grafts which developed spots on the rear end was much smaller than 83 per cent, the reason for this being that when the grafts were taken from the larvae, they did not always include the area where the spots mentioned above are located.

The technique of operation was exactly the same used in former experiments and may be learned from previously published papers on these experiments.³ It should be mentioned however, that while front and rear end of the graft were left unchanged, each piece of skin was turned upside down on its new host, as can be seen from the sketch in figure 1. In the first six experiments the animals were kept in an icebox at 0° to 3°C. before operation. All other animals were kept in a dark cool-room at a temperature of 14° to 18°C. before operation. Immediately after operation each animal was placed in a petri dish, the bottom of which was covered with moist filter paper and a thin layer of water, and placed in a dark incubator at 16° to 18°C. for about twelve to sixteen hours. Then each one was put into a white glass jar holding about 2000 cc.; the jars were filled with tap water and placed in a dark cool-room at an average temperature of 15°C. where the animals were kept permanently.

When the animals should be taken out of the water they show a rather definite and uniform behavior. First they come to the surface of the water in order to change the air contained in the lungs; soon after this they begin to float permanently on top of the water and do not take food for several days. The gills and fins start to

³ E. Uhlenhuth, 1912, p. 735.

reduce in size and then the animals suddenly shed their skin and in the short space of a day or over night, the gills are reduced to mere small filaments. If the animals are not removed from the water at this moment they soon become stiff from asphyxiation, but may recover if taken out before dead. When removed from the water each animal was transferred into a glass jar of the same size as those used for the larvae, but the bottom of the jars was covered with a piece of moist filter paper and a small amount of water. The jars were kept closed all the time by means of plate-glass. In the following text this condition will be called 'land-condition.' This equipment was not changed until the beginning of 1917, when the filter paper was replaced by gravel.

As long as the animals were larval they were fed on *Tubifex* thrown into the water. After metamorphosis earthworms were fed. Though most of the animals after they had been metamorphosed commenced to take readily pieces of earthworms from a pair of forceps, they later on stopped doing this after they had been neglected for some time and had to be fed forcibly by stuffing the pieces into their mouths. It appeared however, that this method of artificial feeding was not satisfactory and many of the animals were lost. It should be mentioned that by feeding the animals regularly they easily learn to take even whole earthworms from the forceps and there is no difficulty in raising Salamanders to full size in this way as we have experienced in other experiments.

Forty-six larvae of *Amblystoma punctatum* were operated upon in the manner described above, an odd number and the consecutive even number making one pair X and Y, each of which carried on one of its shoulders a piece of skin and one eye taken from the head of a third animal A (fig. 1). The animals used for these experiments hatched between the 3rd and 8th of May, 1916, from eggs collected by Mr. R. Deckert of Bronx Park in swamps near White Plains, N.Y., during the spring of 1916. All three individuals of one pair (A, X, and Y) were usually hatched on the same day. Whether or not their origin was from the same mother was not noted. The operations were performed from the 6th to the 22d of July, 1916. The results of this series (Series XXV) are described in the following pages.

2. *The Results.*

a. The Metamorphosis of the Skin Grafts.—Not all 23 pairs gave conclusive results, as was to be expected from the character of the experiments. In six pairs one of the two individuals died before metamorphosis; in six other pairs the development of those characteristics used as indicators of metamorphosis showed some irregularities which prevented final conclusive results. In the remaining eleven pairs the result was conclusive. According to this the experiments may be divided into three groups.

1. In six pairs one animal died a short time after the operation, the remaining animal was nevertheless watched and it was observed that with one exception (experiment 13) where no result was reached at all, the development of the graft proceeded simultaneously with the development of the host.

In experiment 1 the animal was set on land-condition on September 1, 1916;⁴ the first greenish spot on skin graft appeared on September 2; the network of the host was separated into greenish spots on September 5. In host and graft the spots assumed more definite outlines on September 18 and turned yellowish on October 2; on October 3, host and graft appeared cinnamon after having become gradually darker and darker. Both were still darker on October 31; no further examination could be made as the animal died.

In experiment 3, host and graft both turned gradually darker and darker until the animal was set on land-condition on September 7. On September 9, host and graft had developed green spots.

In experiment 6 the yellow network of the host had formed on September 11, while on graft no network ever was developed. The animal was set on land-condition on September 23 and October 9 the network of the host had separated into greenish spots and simultaneously on graft two yellowish spots had come out.

⁴ All observations and notes from the end of July to October 2 were made by Mr. N. Anderson with the aid of a detailed plan given to him for this purpose. All observations and notes previous and subsequent to this period were made by the author himself. On October 3 the results found at this time were compared with Mr. Anderson's notes and were found to be in accordance with them.

In experiment 7 the yellow network of the host appeared on August 28, while on graft there was never any network formed. On August 30 the network of the host was already separated into greenish and yellowish spots and on graft, 5 small spots had formed; the animal was transferred to land-condition the same day. On September 5 host and graft were already very dark and cleared up gradually until they were finally black and their spots bright yellow.

In experiment 13 the host was one of those animals which for some reason do not develop any spots at all on the back or form only after a long time a few faint and small greenish spots, consisting of very loose, tiny greenish or yellowish pigment dots. For this reason this experiment appears to be of very little value in connection with the problem of metamorphosis. Since we will have to come back to this experiment when other problems will be at issue, it may be briefly outlined here.

On August 28 the graft had developed two light yellow spots, while the host showed only a very faint greenish pigmentation spread over the yellow brown background. After the animal was set on land-condition (September 1), host and graft turned darker and darker, but while the graft still had two large greenish spots, the host had remained in the network stage and its network was of such a faint and indefinite character that it could not be traced exactly nor ascertained whether or not it was isolated. The spots of the graft turned bright yellow on September 11 while on the host's body the first greenish spots were not recognized before September 18 and did not become distinct before September 23 (three weeks after the animal had left the water). The body of the host never developed more than a few small greenish dotted spots, but turned black simultaneously with the graft, whose spot finally disappeared.

In experiment 18 host and graft had become quite dark yellow brown on August 28 and on this day both had developed greenish spots. After the animal was set on land-condition (September 1) host and graft turned continuously darker and their spots grew more yellow. Finally, when the spots of the host began to brighten up the graft lost its spot, but its background changed simultaneously with the host into a clear black.

Though in five of these experiments—the sixth will be referred to later—the graft metamorphosed simultaneously with the host, the result is of course, not conclusive, as none of these grafts was controlled by the other animal of the pair, which carried the other half of the skin of the head of animal A; it could therefore be possible that the animal A from which the grafts were taken would have metamorphosed—if living—at the same time as the respective new hosts. Hence the time of metamorphosis in the grafts might have been determined by the animals from which the grafts were taken and not by the new hosts. On the other hand, the results obtained by these experiments are not contradictory to the idea that metamorphosis of the grafted organs is governed by the body of the host and might be even explained on the basis of this rule in case a number of other conclusive experiments could be shown to prove the existence of such a rule.

2. In 6 pairs (experiments 15 and 16, 19 and 20, 23 and 24, 29 and 30, 35 and 36, 37 and 38) both animals lived long enough to metamorphose, but the results were not conclusive for some other reasons. In some of them either the graft or the host did not develop spots, while in others both hosts metamorphosed at the same time, and still in some other experiments the observations made during the development of the skin were not complete enough.

In pair 15 and 16 the graft of experiment 15 did not develop either network or spots; nevertheless it developed simultaneously with the host as indicated by the notes on the changes of the background, and after metamorphosis cleared up into black at the same time that the host's skin did. In experiment 16 the notes on the development of network and spots are incomplete and as the animal died soon after being set on land-condition it was not possible to watch the process of clearing up.

In the pair 19 and 20 the grafts of both animals did not develop spots and also did not show any network, but in both animals the changes of the background progressed simultaneously. When the animals died (September 19) they were preserved in formalin; from the formalin specimens it can still be seen that in both animals graft and host are at the same stage of development. Unfortunately, as both hosts had developed at the same rate, this pair does not permit

of any conclusions concerning the determination of the metamorphosis of the grafts.

In pair 23 and 24 both grafts were spotless pieces of skin. They nevertheless developed a network; but as both animals X and Y formed their network at the same time the grafts also developing simultaneously with their respective hosts, also formed the network at the same time and there was no way of knowing whether the formation of the network was determined by the hosts X and Y or by the animal A from which both grafts were taken. In experiment 23 the graft developed a little spot underneath the eye just at the same time that the host's network separated into yellowish green spots. This little spot soon disappeared. In experiment 24 however, no spots developed at all except a faint little patch of tiny pigment dots believed to be seen for only two days on the hind corner of the graft, about 1 month after the host's network had separated into spots. It is however impossible to draw any conclusions from these 2 experiments.

In experiments 29 and 30 the graft of animal X (experiment 29) never developed any spots, while the graft of animal Y never formed a network; so there was no way of comparing the two experiments with each other. It may be mentioned however, that in experiment 29 the network of the host appeared simultaneously with that of the graft (August 14) and that in experiment 30 host and graft formed their spots at the same time.

In the pair 35-36, network and spots of host and graft were formed simultaneously in experiment 35, but the graft of experiment 36 was a piece of skin which did not develop spots; the notes about the formation of the network of experiment 36 are incomplete.

In experiment 37 where the spots of host and graft formed simultaneously, the notes about the network are missing, while in experiment 38, where the network was formed simultaneously in host and graft, the latter never developed any spots, thus making comparison impossible.

These 12 experiments do not permit of any conclusions as to whether the metamorphosis of the skin actually is determined by the body of the host, neither do they prove anything to the contrary.

3. Twenty of the remaining experiments forming ten pairs were so complete as to correspond in every respect to the plan designed

before the experiments were started and as will be found, these experiments actually support the idea that was outlined in the first pages of this article. The eleventh pair should rather have been placed in the second group but for some reasons, which will be understood later on it was deemed to be convenient to describe the experiments 11-12 under this group.

DATE	EXPERIMENT 21	EXPERIMENT 22
August 7	Host and graft even light brown	Host and graft even light brown
August 14	Host and graft have developed a greenish network	Host and graft have developed a greenish yellow network
August 14	Land-condition	Land-condition
August 30		On host network separated into dark green spots; on graft 3 yellow spots have formed, 2 of which are very bright
September 2	On host network separated into dull yellow spots; on graft 3 very bright and large yellow spots have developed	
December 14	Host and graft have become black step by step. Only 1 spot has remained on graft, which still is far brighter than any of the spots of the host; it is almost orange yellow, while the spots of host are light yellow. The graft's spot is 3 times as large as any of the spots of the host	Host and graft have become black step by step. Only 2 spots have remained on graft, 1 of which is twice as large as any of the spots of the host. Both are still much brighter than the spots of the host and almost orange yellow, while the spots of the host are light yellow. But there is 1 spot in front of the graft on the head of the host which is as bright as the spots of the graft and of the same color as these spots

For each pair a brief abstract from the records collected in the progress of the work is given here.

In experiments 21-22, the hosts developed in the beginning at the same rate, and so did the grafts. Later on experiment 22 got somewhat ahead and developed the spots three days before experiment 21 and consequently the graft of experiment 22 also developed its spots three days before the graft of 21 did, though both grafts were

taken from the same animal. We must admit, however, that not too much weight can be laid upon this result; in the first place the animals were examined only twice a week and the appearance of the spots on the graft of experiment 21 might have been reported three days later than they actually formed; in this case the spots would have formed simultaneously with the spots of the other graft. Secondly it takes several days as a rule for the spots everywhere on the head and body to become isolated, so that some may appear isolated a few days earlier than others even in the same animal; in this case the difference between the grafts might have been caused not by their hosts but by the animal A to which they both originally belong.

DATE	EXPERIMENT 41	EXPERIMENT 42
August 7	Host and graft even yellow brown	Host and graft even yellow brown
September 5	Host and graft have developed the greenish network	Host and graft have developed the network
September 9	Land-condition	
September 11	Host and graft becoming darker; in both network has isolated into large irregular green spots	
September 16		Host's network separated into spots in several places; on graft 2 greenish spots are formed

In both grafts the spots from the very beginning of their appearance were much brighter than the spots of the host; this however, is in no way due to a difference between the rate of development of host and graft but must be explained on the ground of a specific difference existing between the spots of animal A from which both grafts were taken and the spots of the animals X and Y; only this assumption can explain the fact, that the same characteristics appeared in both grafts alike and were retained permanently, since the spots were even more unlike the host's spots after the animal was fully metamorphosed (figs. 7 and 8). We shall return later to this phenomenon. It is more difficult to understand the origin of the orange yellow spot in front of the graft of experiment 22, as the history of this spot is missing. But it seems certain that this spot either belonged to the graft or was formed at least under its influence and with the aid of

the graft's yellow chromatophores. In favor of the suggested origin of this spot is the fact that among a very large number of metamorphosed animals which did not have pieces of skin grafted from other

DATE	EXPERIMENT 25	EXPERIMENT 26
August 7		The first traces of a light yellowish green network have appeared on host and graft
August 14	Host and graft show first traces of a green network	
August 22	On the host the network assumes a more distinct character, while in graft it has become less conspicuous	
September 5	In the host network has started to separate into yellow spots, while in graft only a faint trace of network is left and no spots are formed	
September 8	Land-condition	
September 9	Host and graft cinnamon	
September 11		The network of the host has differentiated distinctly from the brown background and has assumed a yellowish green color. On the graft network has spread its grayish green pigmentation and left only 2 brown lines of the background uncovered
September 15		Land-condition
September 25		On host network has started to separate into large green spots, on graft 1 spot has formed in hind-corner
September 28		Host and graft cinnamon
October 7	Host and graft dark cinnamon	Host and graft very dark cinnamon

individuals, such a wide and very distinct difference in the color of the spots was never observed.

In experiments 41-42, the grafts developed their network simultaneously with each other, as also did the hosts, but the host and graft of experiment 41 developed spots five days earlier than host and graft of experiment 42.

DATE	EXPERIMENT 31	EXPERIMENT 32
August 14	Host and graft show the first appearance of a greenish network	
August 28		On host and graft a grayish network begins to appear; on host it is more of a greenish shade
September 13		Land-condition
September 14		On host and graft yellow spots have separated from network
September 17	Land-condition	
September 23	On host large irregular green spots have formed from the network; on graft 1 large greenish spot has developed right behind eye	

In experiments 25-26, the development of the network on the grafts occurred simultaneously with their respective hosts, but not simultaneously with each other. Hence just the opposite occurred of what would be expected if the rate of development of the grafts were determined by animal A, to which both grafts originally belonged. In experiment 26 the first trace of the network appeared seven days earlier than in experiment 25. In experiment 26 the graft developed all other characteristics at the same rate as its host; in experiment 25 the graft was a spotless piece of skin and cannot be compared further with the host with regard to spot development, but its background changed simultaneously with the host.

In experiments 31 and 32 the network as well as the spots in both grafts developed simultaneously with their respective hosts, but not simultaneously with each other. In experiment 31 the network of the host and graft was formed fourteen days earlier than in host and

DATE	EXPERIMENT 9	EXPERIMENT 10
August 28		First yellow spots appearing on host's skin and 1 on rear end of graft
August 31		Land-condition
September 7	Green spots appear on host and graft	
September 9	Land-condition	

graft of experiment 32, while the spots separated nine days later than in experiment 32. This result can be understood if we assume that the development of network and spots of the grafted skin was determined by the new hosts.

In experiments 9-10, no notes were made on the development of the network. The spots of host and graft developed ten days earlier in experiment 10 than in experiment 9.

DATE	EXPERIMENT 39	EXPERIMENT 40
August 14	On host and graft first traces of greenish network have developed	On host and graft first traces of a greenish network appear
August 28		
August 30	On host first greenish spots have isolated from network. On graft only in center a part of the greenish network still present	
August 30	Land-condition	
September 6	On host network everywhere separated into large irregular green spots. In graft network disappeared, no spots developed	It is only now that the green network of the host and graft has become distinct
September 11		
September 15		
September 23		
December 21	Host's spots almost orange-yellow, graft has no spots	Host's spots almost orange yellow; graft with 2 light yellow spots

In experiments 39-40, the network of the grafts again developed as if the rate of development would be determined by the respective hosts. In graft and host of experiment 39 it appeared fourteen days earlier than in experiment 40, and in experiment 40 it became distinct twelve days after the isolation into spots had started in experiment 39. The development of the spots of experiment 40 occurred simultaneously in host and graft; in experiment 39 the graft was a

spotless piece of skin and comparison of the development of the spots was impossible.

This case is perfectly clear as to network development. It developed simultaneously in each graft and its host, but twenty-one days earlier in graft and host of experiment 46 than in graft and host of experiment 45. The development of the spots however, seems confused at

DATE	EXPERIMENT 45	EXPERIMENT 46
August 7		On host and graft greenish network has developed
August 28	Host and graft have formed a greenish network	
September 12	Land-condition	Land-condition
September 14		Host's network has isolated into green spots; in graft yellow pigment dots, chiefly in center, have developed, but no large spots
September 21	Network of the host has not separated yet, but forms a greenish rather faint pattern, while in graft 1 yellow spot has formed in the rear end	
October 4	Host's spots appeared today	
October 31		Spots of the host bright yellow; graft spotless
December 26	On the body of the host (right side) there are only 3 small and faint light yellow dotted spots, while the spot of the graft is very bright and almost orange yellow	

the first glance in both experiments. In experiment 45 the host developed its spots 11 days after the spots of the graft had developed. We have already mentioned a similar case (experiment 13) and we will find a few more animals acting similarly. All individuals which developed their spots after the graft had developed them, showed one common characteristic when they were fully metamorphosed: they were almost bare of yellow pigment. The host of experiment 45 for instance, developed only very small and faint spots which lacked the

usual compactness of the spots and gave only the impression of an accumulation of little yellow dots on the black background. As their number (on the right side) was only 3 (fig. 9), the animal appeared almost uniformly black. We believe therefore that in this case a factor whose nature will be explained later on, interfered with the metamorphosis factor. In experiment 46 the graft did not develop any real spots, but only a few small dots which appeared simultaneously with the spots of the host, but later on disappeared entirely.

DATE	EXPERIMENT 43	EXPERIMENT 44
August 28	On host and graft first traces of a greenish network have formed	
September 5		On host a greenish yellow network has started to form, on graft the network consists of a faint greyish green pigmentation
September 11		On host several yellow spots have isolated, in graft 1 yellow spot has appeared
September 13		Land-condition
September 30	Land-condition	
October 4	Host's network separated into dim yellowish spots, in graft some yellow spots are developing on rear end	
December 26	Host with bright yellow spots; graft has lost all its spots	Host with large yellow spots; graft has lost its spot

In this case, however, it was impossible to decide whether or not this was due to the graft being a spotless piece of skin as this graft soon after metamorphosis assumed a pathological appearance; it became grayish and edematous and was gradually replaced by the surrounding skin of the host. Therefore the development of the spots in none of the experiments of this pair can be used in studying the problems of metamorphosis.

In experiments 43-44, the grafts developed the network independently from each other and simultaneously with their respective hosts, 43 forming the network of host and graft eight days before host and graft of 44. Both grafts finally became spotless, since the spots developed in the beginning disappeared later on. But these tem-

porary spots were developed in both grafts at the same time as the spots of the respective hosts, in experiment 43, twenty-three days later than in experiment 44.

DATE		EXPERIMENT 27	EXPERIMENT 28
July	31		On host and graft a grayish green network has started to form
August	28	On the host a greenish network has started to develop; on graft a faint green network also is forming	
August	30	The network of the host has separated into bright yellow spots and from the network of the graft patches of yellow pigment dots have developed in the center of the graft, which are less bright than the spots of the host	
August	30	Land-condition	Land-condition
September	5		In host there are no spots, only a network like greenish pigmentation while in the graft only a part of the network is left and in the center 2 distinct yellow spots have formed
September	9		
September	14		In host the network is still present but several yellow spots around the skin-graft have finally developed
October	7	Host's spots still dim yellow, graft's spots disappeared	
October	12	Animal escaped	
December	18		Host's body almost without spots, since only 3 light yellow spots near the shoulder of right side and a few around the grafted skin of the left side have developed.

Experiments 27-28 are perfectly conclusive in regard to the development of the network, which was formed by both grafts simultaneously with their respective hosts, but appeared in host and graft of 27, twenty-eight days later than in host and graft of 28. Concern-

ing the development of the spots we find that the graft of 27 finally became spotless, but developed during metamorphosis a few tem-

DATE	EXPERIMENT 11	EXPERIMENT 12
September 5	On graft 2 pale spots are developed. On host there are no spots as the network has not yet separated	In graft and host the network is formed
September 8		Land-condition
September 9		The network of the graft has separated into 2 yellow spots, the network of the host has not yet separated
September 11	On graft 2 more spots have developed. On host very faint and fine yellow pigment dots appear occasionally	
September 13	Land-condition	Host's network separated into faint green spots; graft's spots bright yellow
September 20		In front of the grafted eye 1 bright yellow spot has developed on the graft
September 22	On the host several faint spots can just be traced	
September 23	The spot of the graft appears very bright compared with the spots of the host	
December 13	Host only with a few pale, very small dotted greenish spots, graft with 1 very bright, almost orange yellow spot	Host only with a few pale, very small dotted greenish spots (none on head and only 1 on right side of body). Graft with 2 pale dotted spots and 2 very bright, almost orange yellow spots, 1 of which in front of eye, cannot be determined now as lying on the graft, because the graft's borders have disappeared but its history shows that it developed on the graft

porary spots simultaneously with the host. In experiment 28 we have before us a case which is similar to that of experiments 13 and 45. The network of the host did not separate when the network of the

graft loosened up into spots, but needed five days longer for this process and even then had not entirely disappeared. When the animal had completed metamorphosis it turned out to be again one of those individuals whose skin is almost lacking in yellow pigment; only a few light spots had formed on each shoulder (fig. 10). This case will be discussed together with experiments 13 and 45 later on.

In experiments 11-12 we have a case similar to experiment 28 which will demonstrate particularly well the difference between the factor that is concerned in this kind of delayed development of the yellow spots and the factor which is involved in metamorphosis, for in this case both hosts were of this almost spotless variety and both behaved towards the grafts in a similar way. In addition to this, the grafts were taken from an animal whose spots were characterized by a particularly vivid and almost orange yellow coloration as shown by the fact that both grafts showed this feature in the same way. Since both characteristics are permanent even after metamorphosis (figs. 11 and 12) they apparently must be parts of the specific individuality of the adult animal, which of course, cannot be changed by the action of the metamorphosis factor, but on the contrary should be developed by this factor.

In the experiments mentioned until now the difference between the rate of development of the two grafts was rather slight, ranging from seven to twenty-eight days in regard to the network and from three to twenty-three days in regard to the spots. This was apparently due to the fact that the respective hosts did not show any greater differences. The last pair of experiments, however, will prove that the difference between the rate of the grafts increases in exactly the same way as the difference between the rate of development of the respective hosts increases and that one graft may metamorphose very much earlier than the other one as soon as one succeeds in accelerating metamorphosis of one of the hosts and in delaying metamorphosis of the other host a considerable length of time.

In this pair of experiments one animal (experiment 34) did not metamorphose at all, though it was kept under apparently exactly the same conditions as experiment 33 and as all other specimens described until now. On January 10 it was subjected to another operation in consequence of which it died; at this time the animal still did

not show any signs of metamorphosis nor did its skin graft. Host and graft were of an even coloration and no spots or network had developed. Host and graft of experiment 34 were in larval condition

DATE	EXPERIMENT 33	EXPERIMENT 34
July 19	Operated	Operated
August 7	On host and graft the first traces of a greenish network are already visible	Host and graft even yellow brown
August 28	On host and graft a distinct network has developed	
September 8	Land-condition	
September 11	Host's network separated into yellowish green spots; on graft 2 large yellow spots have developed which are brighter than host's spots	
September 25	Spots of the host greenish yellow; spots of the graft very bright yellow	
October 10	Photographed and painted in natural size	Photographed and painted in natural size
October 19		Photographed in four times the natural size and painted
<i>1917</i>		
January 10		Host and graft still even yellow brown (no network or spots), both being in larval condition
January 10		Dead (killed by a second operation)
		Preserved in formalin
January 30	Dead (preserved in formalin)	
February 18	Photograph of October 10 enlarged and painted with the aid of the notes, the formalin specimen and some living animals, which were similar in color to experiment 33	

five months after the other graft—though taken from the same animal A as the graft of experiment 33—and its host had developed the first traces of the network and four months after they had developed their spots.

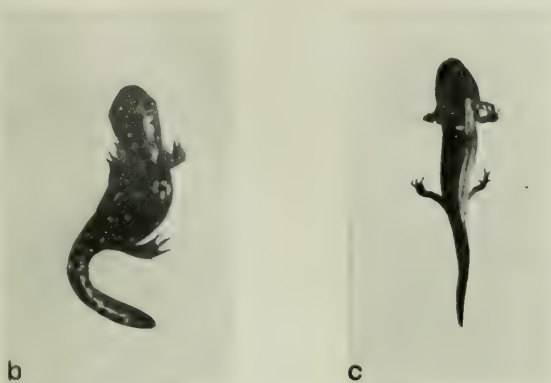
This will be illustrated best by the photographs and pictures which

were made from both experiments. Both experiments were photographed in natural size on October 10. Text figure *b* shows experiment 33 fully metamorphosed and the spots developed all over the

DATE	EXPERIMENT 33	EXPERIMENT 34
September 8	Land-condition	Larval-condition
September 29	Today the grafted eye was examined for the first time, but the eyes of the host were not examined. The yellow ring of the grafted eye is broken up into black and yellow dots	Host's eyes as well as grafted eye still possess a plain, continuous yellow ring
October 17	Today the eyes of the host were examined also. In all three eyes, the two eyes of the host and the grafted eye, the yellow ring is broken up into yellow dots	The rings of all three eyes are free from any black pigment
December 19	In the iris of both eyes of the host a number of very tiny yellow dots are still present, while the rest of the iris has become black. In the iris of the grafted eye a small number of yellow dots are still present, the rest of the iris is black	The inner section of the iris of the grafted eye appears, even with a larger magnification of a binocular lupe to be a continuous yellow ring, in which no melanophores are detected. The outer section contains a large number of yellow chromatophores, which are darker than the other yellow chromatophores and more reddish, and contains several parts in its course which are made up by grayish-blue cells. Both eyes of the host are in exactly the same condition as the grafted eye
1917		
January 10		All three eyes still in the same condition as when last noted
January 10		Killed by a second operation

body. Behind the eye four spots are visible; two of them, lying behind each other in a line parallel to the longitudinal axis of the body, are only highlights produced by the reflection of the light on the black shining skin of the animal. The other two lighter spots are two yel-

low spots belonging to the graft. Just in front of the bulb of the grafted eye a third small spot is situated which also belongs to the graft. All these spots were almost orange yellow, thus being distinctly different from the spots of the host; the two spots in back of the grafted eye were considerably larger than any of the spots of the host, which fact is well shown in the picture. Text figure *c* shows experiment 34 photographed on the same day as experiment 33. This animal was still larval at that time, as the gills are still present and no yellow spots are developed. In accordance with the condition of the host the graft, whose borders can be only partly distinguished in this photograph, has no spots also.



TEXT FIGURES *b* and *c*.

Nevertheless these photographs were not considered entirely convincing since they lacked detail, in addition to which they also could not demonstrate the rather minute details of the eye which will be needed later. Experiment 34 was therefore photographed again on October 19, and this time in four times the natural size; the photograph was then painted carefully. This is shown in figure 13. The borders of the graft can now be seen quite distinctly, the graft is still the even color of a larva of *A. punctatum*, but is slightly darker than the host. The dark patch in front of the eye corresponds to a tumor-like elevation of the grafted skin produced probably by a proliferation of the epithelial cell, such as is often found in examining skin grafts

of Amphibians on microscopic sections. The photograph of experiment 33 taken on October 10 was enlarged on October 19 but was not painted before February 18, 1917 (fig. 14) when the animal was already dead and preserved in formalin. In the period from October 10, 1916, to January 30, 1917 (the date the animal died) the two spots of the graft in back of the eye and particularly the spot in front of the eye, increased somewhat in size; the size of these spots in figure 14 corresponds to their size on January 30, 1917, as they were drawn from the preserved specimen. The picture (fig. 14) shows also that besides the difference in size there is a considerable difference in the color of the graft's and host's spots. When the picture was made the colors were painted according to the notes made during the observations of the living specimen, with the aid of other living animals whose color was known to have been similar to experiment 33, and partly from memory; but it is believed that the picture is in color at least, very close to the actual colors of the living specimen. In the preserved formalin specimen all spots are of course, much bleached but nevertheless the difference between the color of the spots of the host and graft can be seen most clearly.

b. The Metamorphosis of the Grafted Eye.—As previously mentioned no special attention was paid to the metamorphosis of the eye in this series of experiments on *A. punctatum*. Only in one pair of experiments (33-34) described in the last chapter, the metamorphosis of the eyes was watched during part of the life of the animal, so as to give a fairly continuous series of observations. An abstract from the notes is given here.

From these observations it is evident that in experiment 34 the ring of the grafted eye was still unbroken, about three months after the yellow ring of the grafted eye of experiment 33 was broken up into black and yellow dots. The same relation existed between the eyes of the respective hosts. The condition of the grafted eye as it existed on October 19, may be seen in figures 13 and 14; this condition was only little changed, when the animal died. Hence, in experiment 34 metamorphosis of the grafted eye could not take place because the host did not metamorphose, while in experiment 33 the grafted eye, though belonging originally to the same animal A as the grafted eye of experiment 34, was forced into metamorphosis by its host at least three months earlier than experiment 34.

B. Heteroplastic Transplantations from Larvae of Amblystoma punctatum to Amblystoma tigrinum.

The object of these experiments was to ascertain whether or not organs of one species could metamorphose on the body of another species. Eyes and pieces of skin were grafted from *A. punctatum* to *A. tigrinum*. The experiments actually showed that eyes as well as skin are able to metamorphose even when grafted to another species, and that they metamorphose at the time that the host metamorphoses.

1. Material and Method.

The technique of operations employed in these experiments (series XXVI) was the same as that used for series XXV. The skin of one-half of the head including the eye was taken off from a larva of *A. punctatum* and grafted to the region just back of the shoulders of a larva of *A. tigrinum*, after a piece of skin the size of the graft and a small amount of tissue of the *musculus longissimus dorsi* had been cut out from there.

The general method was somewhat different from series XXV, as the experiments were not arranged in pairs; the animal A from which the graft was taken being a different individual for each experiment. The left eye of individual A (*A. punctatum*) was grafted to individual X (*A. tigrinum*), X being the experimental animal which alone was kept alive and examined from time to time.

It was of course, impossible to compare the developmental stages of the grafts with the developmental stages of the host with such exactness as in series XXV, since the changes of the organs of *A. tigrinum* are entirely different from those of the graft. Consequently only one definite step was left to which to refer the changes of the grafted *punctatum* organs, and that was the moment when the *tigrinum* host leaves the water. Doubtlessly, a certain amount of inaccuracy enters these experiments, for different individuals may show a different degree of development of some of their characteristics when they leave the water, a condition frequently noted during observation of a large number of Urodelan larvae. We shall see, however, that the difference in the dates when the hosts left the water

is wide enough to establish a corresponding difference between the grafts and to make the relation between host and graft a definite one.

Furthermore, it must be said that it was not known exactly when the grafts would have metamorphosed if they had remained with individual A, for this individual had been killed and could not be used as a standard. But an approximate normal standard-time of the metamorphosis of the graft can be calculated from the records made about the time of metamorphosis of the entire stock of *A. punctatum* larvae from which our grafts were taken. According to these notes, from approximately 300 *punctatum* larvae all but about 10 metamorphosed before October 1, 1916. Four of the five grafts to be reported also metamorphosed before this time, together with their hosts; the fifth metamorphosed late in October and later than any *A. punctatum* larva of the entire series of experiments described in this paper. As the respective host showed a corresponding difference, compared with the other hosts, it seems justified to conclude that in all probability the time of metamorphosis of the grafts in this series (XXVI) was also determined by the hosts.

The material used as grafts was the same as used in Series XXV; all animals were hatched on May 6 and were in this case the offsprings of the same mother. The tigrinum larvae were collected in the early part of July, 1916 in ponds on Long Island by Mr. Deckert and myself.

The operations were performed on July 21 at which time the tigrinum larvae ranged from 57.00 mm. to 87.50 mm. and still possessed truly larval characteristics: their gills and fin were of enormous size, the color of the skin was still yellowish and the melanophores had not yet formed any definite pattern.

All animals were kept, before and after operation, in a cool, dark room at an average temperature of 15°C.

2. The Results.

Among the seven experiments of series XXVI only five were made in the manner described above; in some of them the eyes, in others the skin, and in still others both of these organs were examined. Abstracts from the records follow.

Experiment V. August 28, 1916. On skin graft network has separated into large greenish spots, which are entirely different from the light sepia spots just developing on host's skin; in this way it is very easy to identify them as Punctatum spots.

September 2. *Host on land-condition.*

September 5. On skin graft 3 yellow spots of the punctatum type have formed definitely.

September 22. The spots on skin-graft are now bright yellow.

September 25. Only 1 yellow spot of skin-graft left, which is now still brighter than before.

October 3. The grafted punctatum eye, which has not been examined until now, shows its ring broken up into yellow and black dots.

December 7. The 1 yellow spot on skin-graft has become very faint.

Experiment III. September 7. Yellowish spots and dots of indefinite number and appearance are developing on skin graft.

September 22. Two grayish and 1 greenish yellow spot have assumed full distinctness and a small patch of yellowish pigment dots has formed.

September 22. *Host on land-condition.*

September 24. All spots of graft have become greenish yellow, they are distinctly to be recognized as punctatum spots and are absolutely different from the grayish sepia spots of the host.

September 29. The patch of yellow dots on the graft has developed into a single spot of the punctatum type and is extremely bright yellow.

October 4. Only 2 of the yellow spots on the rear end of the graft are still present.

October 26. One of these 2 spots has entirely disappeared, the other has been replaced by the chromatophores of the host and has turned sepia.

November 23. All spots of the graft have gone.

Experiment VI. Skin-graft started soon after the operation to be replaced by host's skin.

September 20. In the grafted punctatum eye the outer section of the yellow ring shows dark places.

September 23. *Host on land-condition.*

September 30. Whole ring of grafted eye broken up into yellow and black dots.

Experiment VII. September 25. On skin-graft, 1 yellow spot of the punctatum type has developed. Grafted eye with dark places in the outer section of the yellow ring.

September 29. The yellow spot of the skin-graft has turned brighter.

September 29. *Host on land-condition.*

October 4. On skin graft 3 more yellow spots of the punctatum type have formed. Entire ring of grafted eye broken up into yellow and black dots.

October 10. Photographed and a few days later painted (fig. 16); compare with figure 15 of experiment IV.

October 16. On skin-graft all 4 punctatum spots still present, but 3 of them have become less dense. Only a few yellow dots are left in the grafted eye.

December 7. All 4 spots of the skin-graft have become considerably fainter, but are still present and of the punctatum type. No yellow dots left in the grafted eye.

Experiment IV. October 4. Host still in a larval condition. The ring of the grafted punctatum eye is still an unbroken continuous line.

October 10. Drawn and painted (fig. 15); compare with figure 16 of experiment VII.

October 26. Outer section of the ring of the grafted eye shows dark parts, inner section an unbroken yellow line.

October 27. *Host on land-condition.*

October 31. Ring of the grafted eye broken up into yellow and black dots.

November 20. Still a small number of yellow dots in lower half of iris of grafted eye present.

November 20. Photographed.

November 24. The photograph of November 20 is painted after the living animal which has not changed since that time (fig. 17).

In all five experiments the grafted organs of *Amblystoma punctatum* underwent changes typical to metamorphosis of these organs about the time that the tigrinum hosts left the water. The changes of the grafted skin were stretched, as under normal conditions, over a longer period, beginning somewhat before and ending somewhat after the host had left the water. Comparing the date of appearance of definite spots on the skin-graft with the date of the host being set on land-condition, we notice a very definite time-relation between these two phenomena: the later the latter occurs the longer the former is delayed.

	APPEARANCE OF DEFINITE SPOTS ON GRAFT	LAND CONDITION OF HOST
Experiment V.....	August 28, 1916	September 2, 1916
Experiment III.....	September 22, 1916	September 22, 1916
Experiment VII.....	September 25, 1916	September 29, 1916

Much more definite in time is the breaking up of the inner section of the yellow ring into black and yellow dots, as it never occurs before the tigrinum host has left the water, a relation which is in perfect accord with the attitude of the large majority of normal punctatum eyes. And again, the more delayed the host's going on land, the

later the breaking up of the ring occurred. In experiment 5, the eye, unfortunately was not watched when the host left the water, but one month later when the eye was examined for the first time, it showed the ring broken up into black and yellow dots. Even if we assume this to be the date of actual breaking up, this eye would have undergone the change of its yellow ring almost one month earlier than the eye of experiment 4, whose host correspondingly left the water two months later than the host of experiment 5.

In experiment 4, the host and the graft were still in larval condition when the hosts and grafts of the other 4 experiments had metamorphosed. Since in this case the punctatum graft metamorphosed far later than any *A. punctatum* larva in the experiments reported here, this experiment had particular interest. On October 10, experiment 4 was pictured (fig. 15), and for comparison experiment 7 was pictured (fig. 16).

	HOST SET ON LAND- CONDITION	BREAKING UP OF THE YELLOW RING OF THE GRAFTED EYE
Experiment VI.....	September 23, 1916	September 30, 1916
Experiment VII.....	September 29, 1916	October 4, 1916
Experiment IV.....	October 27, 1916	October 31, 1916

Figure 15 shows the host in a perfectly larval condition, the gills and the fin of the tail still being very large. The grafted eye, which can also be seen in the picture still possesses a bright yellow ring in place of the iris; no black pigment can be detected. In figure 16, which is a painted photograph of experiment VII, the host is seen to be metamorphosed and has developed the pattern of the metamorphosed young *A. tigrinum*. The graft also is metamorphosed, as is demonstrated by its dark almost black background color and by the lemon yellow spots which it developed. The grafted eye appears very different from experiment IV; it has lost its yellow ring almost entirely, only a few yellow dots remaining. The grafted eye of experiment IV did not reach this condition before the middle of November, when it was photographed and painted again (fig. 17). Only a small number of yellow dots were still present.

Thus it seems impossible to overlook in these experiments a definite time relation between the metamorphosis of the host and that of

the grafted organs, which evidently means that the organs of *A. punctatum* cannot metamorphose before the host does and that they are forced into metamorphosis as soon as the host metamorphoses. The ability of one species to bring into metamorphosis the organs of another species, is the result which we wish most to emphasize among the facts disclosed by this series of experiments.

IV. DISCUSSION.

1. The experiments reported in this paper seem to throw some light on certain fundamental principles upon which the mechanism of the metamorphosis of the Amphibian skin is based.

Let us consider first the experiments of series XXV in which from the head of one individual each of the two halves of the skin was grafted to another individual of the same species and the rate of development of the yellow pigment spots of the adult was compared in each graft with the rate of the same process in the respective host and the other graft. Under normal conditions, i.e., if the two pieces of skin had remained with the individual to which they originally belonged, both pieces would have developed the yellow spots at the same time and would have metamorphosed simultaneously with each other. If all the factors necessary to the development of the yellow spots were in the skin itself, i.e., if this phenomenon were actually a process of self differentiation as Weigl claimed it to be, there would be no reason why two pieces of skin of the same animal should not develop simultaneously with each other even after they have been separated from this animal and transferred to different individuals.

The two pieces however, did not act in this manner. On the contrary:

a. Pieces of skin grafted from one larva to another developed the network and the yellow spots simultaneously with the host instead of with each other; i.e., they developed the characteristics of the adult skin only when the host developed them, and

b. This resulted in the establishment of a more or less considerable difference in the time of development of the adult characteristics between the two pieces of skin taken from the same animal provided

such a difference existed between the two respective hosts. This difference ranged from seven days to five months with regard to network development, and from three days to four months with regard to the development of the yellow spots (separation of the network).

Such a relation can exist only if at least one factor necessary to the development of the adult characteristics of the skin is not present in the skin itself but must be furnished to the skin from the host. This factor which may be called "metamorphosis factor of the skin" is a factor external to the skin, and metamorphosis is not a process of self differentiation, for it is dependent on a body (host) which produces this factor.

It appears therefore, that in the metamorphosis of the skin a factor has manifested itself which is similar to the metamorphosis factor of the eyes and of the gills of *Salamandra maculosa*, for the metamorphosis factor of all three organs is necessary to induce metamorphosis in these organs and is primarily not contained in the organs but in the body of the organism. From experiments 33-34, of series XXV, it appears that the eye of *Amblystoma punctatum* also follows this mode of metamorphosis.

According to the method employed, the experiments described in this paper could not be expected to furnish any evidence concerning the question as to whether the second and third quality of the metamorphosis factor of the eye of *Salamandra maculosa* mentioned on the first page of this article, will be found again in the metamorphosis factor of the skin of *Amblystoma punctatum*, although this assumption seems justified. As to the fourth characteristic of this factor, we have obtained a definite answer in the experiments of series XXVI on heteroplastic transplantation. Pieces of skin and eyes of *A. punctatum* when grafted to another species (*A. tigrinum*), showed exactly the same mode of metamorphosis as they showed when grafted to an individual of their own species. Metamorphosis of these two organs did not take place unless and until the host metamorphosed. After we have seen that neither of these two organs contain the metamorphosis factor, this can only mean that the factor has been furnished to them from the body of an individual of a foreign species. Hence the factor in question must be 'non-specific.' It was mentioned on

page 33, that some evidence of non-specificity was also found for the metamorphosis factor of the eye of *Salamandra maculosa*.⁵

2. One objection might be raised against the method employed here. In certain cases of skin grafting the grafted pieces of skin are soon partly or entirely replaced by the host.

If, then, in the experiments of series XXV the skin graft had been replaced by the skin of the host before the appearance of the network or the yellow spots, the yellow spots developed on the place corresponding to the grafted piece of skin, would actually have been spots of the host and not of the graft. It would not be surprising then that these spots always developed at exactly the time that the rest of the spots of the host developed.

Although the fate of the grafted skin of the homoplastic series has not been followed on histological sections, there is enough definite evidence at hand to show that no replacement of the graft took place.

First: the edges of the graft retain complete definiteness throughout the larval period. This can be ascertained as the color of the graft and that of the host show as a rule at least slight individual differences (fig. 13). Later on when the animals are entirely metamorphosed, graft and host are uniformly black and the borders of the graft can no longer be distinguished. This fact does not interfere with our results, however, as at this time the phenomena on which the present investigation is based are long past.

Second: if replacement of the graft actually takes place it is easy to notice it macroscopically, without the aid of microscopic sections. This was demonstrated by the behavior of the graft in experiment 46.

Third: certain individual differences between the yellow spots of different individuals may help in determining the origin of the yellow spots on the graft, as they reappear in the graft even if the spots of the host are of a very different type from that of the graft's spots. In such cases it is easy to distinguish between the spots of the graft and the host. For instance in experiments 11-12, and experiments 21-22, there could have been no doubt that the almost orange yellow spots did not belong to the host but to the graft, even if the history of these spots were unknown. The spots of all four hosts were of a

⁵ E. Uhlenhuth, 1913 c, p. 353.

faint yellow, and orange yellow spots could not be found on their bodies except where the graft had been made. As both grafts of each pair were taken from the same individual we would expect them to develop the same type of spots, which is what actually happened. The results in experiment 33 are similar.

Fourth: Weigl,⁶ who also studied homoplastic grafts of Amphibian skin, had experiences similar to ours concerning the possibilities of replacement of the graft by the skin of the host. According to his statements, the surrounding skin of the host never grows over or into the grafted piece of skin nor does the pigment of the graft spread into the skin of the host nor the pigment of the host migrate into the grafted skin.

The heteroplastic grafts behave very differently from the homoplastic ones insofar as they are actually replaced by the skin of the host, as will be described in another article. It will suffice to mention here that this replacement did not interfere with our experiments since it did not begin before the host had left the water (except in experiment VI). The fact that in some heteroplastic skin grafts, spots of the punctatum type were formed, in itself demonstrates that the grafts were normal at least to such an extent as to produce that phenomenon upon which our conclusions are based. The same is true for the eyes of series XXVI, which were grafted from the punctatum larvae to the tigrinum larvae. They showed themselves as normal by the fact that the breaking up of the yellow ring occurred in a normal way, and as we have selected only this phenomenon for our conclusions, it is not a matter of importance what the condition of these grafted eyes was in regard to certain other histological details. Nevertheless it might be mentioned here that histological examination of the eyes proved that even several months after the breaking up of the yellow ring most of the structures and in some eyes all of the structures were still perfectly normal. Cones and rods were present and unchanged and the stroma iridis, which is the place of the phenomenon in question, showed no divergence from the normal structure of this organ. The only change observed is that the cornea is overgrown by one or more epithelial layers. The course of this

⁶ R. Weigl, 1913, p. 597.

process, however, can be followed macroscopically under the binocular. It did not begin before the ring was broken up and since the epithelial layers are translucent in the beginning and can be kept so for a long period by being kept wet, examination of the iris is still possible after the epithelium of the skin has commenced to grow over the cornea.

3. Concerning Weigl's opinion that metamorphosis of the skin is a process of self differentiation, we do not believe that the results of his own experiments justify such an assumption. In fact it seems that they would appear rather confusing and lacking in uniformity if metamorphosis actually were a process of independent differentiation.

Our experiments on eye grafting have demonstrated that the age of the host as well as the age of the graft—or more correctly, the stage of development of host and graft—are important considerations inasmuch as the metamorphosis factor is not carried into the eye before the host has reached a certain stage of development. If the organ be taken from an individual at this stage, it would of course develop independently from its new host. On the other hand, very young organs for some reasons respond to the action of the metamorphosis factor only after they have been grafted to the new host a certain length of time. Hence it is clear that the true relation existing between the body of the individual and its organs cannot be studied unless attention is paid to age and developmental stage of both of them. This can only be done if the age of each individual is carefully registered and the developmental stage of each animal is recorded in definite terms, and if a special series of experiments is devoted to each of the ages and stages which play a rôle in the phenomena under discussion. Unfortunately such data are missing in Weigl's paper; it is impossible to form any conception of the age and developmental stage of such larvae which Weigl calls 'young' larvae or 'old' larvae. If the experiments are made with the point in mind mentioned above, the results become uniform and point to the conclusion that metamorphosis is not a process of self-differentiation.

Weigl's experiments may be briefly reviewed here in order to show that they do not contradict the existence of a metamorphosis factor.

First: he found that pieces of skin which were grafted from such larvae as he calls 'young,' to other 'young' larvae, always metamor-

phosed simultaneously with the host, which is in perfect accord with our own results, and Weigl himself expresses this fact in terms that can hardly be brought into agreement with his opinion that metamorphosis is a process of independent differentiation, since he says that the graft becomes entirely subjected to the influence of the host.

Second: he found that pieces of skin grafted from young larvae to old larvae metamorphosed some time after the host. He does not say how young the 'young' larvae were in this case nor how old the 'old' larvae were. If the 'young' larvae were only several weeks old and the 'old' larvae old enough to metamorphose a few days after the operation, this result also would be in accordance with our own results. It does not mean, however, that there is any justification for the assumption that in these young grafts metamorphosis occurred independently from the host's body; it only means that the metamorphosis factor must act a certain length of time before metamorphosis in the organ can begin. If the host metamorphoses before this time is elapsed, the grafted organ will metamorphose somewhat later than the host. Furthermore as repeatedly pointed out in this article, only one factor necessary for metamorphosis is furnished to the graft from the host, while the others must be contained or developed by the graft itself and as long as they are not—which may be the case in very young animals—the skin will not respond to the action of the metamorphosis factor. Whether we call the factors contained in the organ itself 'structure' as Weigl did, or whether we imagine them to be something else, does not alter the fact that the final result is brought about with the aid of the one factor contained in the body of the host, as is shown in Weigl's experiments by the fact that the young organs after having been grafted to old larvae, metamorphosed earlier than they would have done when left with the young larvae from which they were taken. Metamorphosis therefore, manifests itself as a process of 'dependent differentiation.'

Third: Weigl reports one experiment in which the skin of a larva which he calls 'young' was grafted to another larva of the same species (*Salamandra maculosa*); the skin graft metamorphosed six months before the host was photographed and the photograph shows distinctly that the host is still a larva. This case could hardly be explained on the basis of metamorphosis being induced by a factor ex-

ternal to the organ, for such a factor could neither have been carried into this piece of skin from the animal to which it first belonged, since this animal was a young larva according to Weigl, nor could it have been furnished to it by the host, since the host was still in larval condition. The fact that among the many grafting experiments which I had occasion to observe such a result was never obtained makes it appear doubtful whether it could be obtained at all, and there are no data even in Weigl's paper itself which explain why he should have obtained such a result only in one case and not in other cases. We have seen that this result was obtained only when the larvae from which the graft was taken were so old as to metamorphose a few days after operation, and this seemed to be in consequence of the metamorphosis factor being already developed in such very old larvae and carried into the organ. It is to be regretted therefore, that there are no statements in Weigl's paper as to when the individual used in the experiment in question had hatched, nor is there any picture or measurement of this individual to be found indicating the appearance of the individuals which Weigl⁷ calls 'young.' Furthermore, although it is true that the photograph leaves no doubt as to the fact that the host was still a larva, there is unfortunately, in this photograph no definite indication that the graft had actually metamorphosed. It seems to us that the records accompanying the paper are not as complete as they should be to lead us to accept this remarkable and only exception among so many experiments.

Fourth: to a young larva of *Salamandra maculosa* a piece of skin from a young larva of the Mexican axolotl was grafted; a short time after the host had metamorphosed the skin graft metamorphosed also. It is well known that the variety of the Mexican axolotl bred in Europe, does not metamorphose and in the case before us the axolotl larvae which were kept as control did not metamorphose even after they were removed from the water and set on land. They did not show the slightest indications of metamorphosis when the grafted piece of skin metamorphosed. Weigl himself cannot but admit that the graft in this case was forced into metamorphosis by the host—

⁷ R. Weigl, 1913, figure 7, plate XXVIII.

even of a foreign species. In the face of this experiment, which is in accordance with the experiments reported by the writer in this article and in a former paper, and which supports the viewpoint held by the writer, it seems difficult to understand how Weigl could defend the idea that metamorphosis of the skin is a process of self differentiation.

If we compare the results mentioned in the foregoing four paragraphs we shall appreciate how much they would lack in uniformity if viewed from the standpoint that metamorphosis is a process of self differentiation; i.e., that all factors necessary to the metamorphosis of an organ are contained and developed by this organ. It seems, however, that all results, including those obtained by Weigl so far as they are based on definite data, uniformly point to the one conclusion: There is in metamorphosis of an organ (i.e., in the development of the adult characteristics of an organ from the larval structure of this organ) one factor involved, which is not contained in this organ nor can it be produced by this organ.

4. In the same direction seem to point the experiments on feeding thyroid-gland to amphibian larvae and on extirpation of the thyroid and hypophysis in amphibian larvae. These experiments demonstrate at least that the influence of certain substances which are introduced or contained primarily in the body of the animal and not in the organs in question, is in some way able to reach these organs; they demonstrate beyond any doubt that metamorphosis can be induced by an external factor not primarily contained in the organs themselves.

It seems interesting to us that a certain resemblance doubtlessly exists between the action of thyroid substance and that of the factor disclosed in the grafting experiments. First, they are both external to the organs and second, both are non-specific. Third, it seems that thyroid, like the metamorphosis factor, acts only by inducing the processes of metamorphosis; if these processes have once been induced by the action of the substance they will continue even in its absence. Kahn, for instance, stated that a single feeding with thyroid is enough to induce metamorphosis and that without any further feeding metamorphosis may be completed in a short time. We have seen that in eyes in which metamorphosis has once been induced it

will be completed even if the eyes are removed from the influence of the agent and transferred to young larvae in which this agent has not yet developed. Fourth, it seems that in very young animals thyroid must act longer than in older animals in order to induce metamorphosis.⁸ This corresponds with the fact that organs grafted from young to old larvae in which metamorphosis would occur a few days after operation, metamorphose sometime after the host instead of simultaneously with the host.

In our experiments it was not possible to demonstrate actually that the metamorphosis of all three organs examined, i.e., eyes, gills and skin, is controlled by one or more common centers, by which the factor in question is produced. The fact however, that certain important characteristics are common to the metamorphosis factor of each of these organs, encourages the belief that we are dealing in each of these organs actually with the same factor. The results shown by feeding thyroid and particularly by the extirpation of inner secretory glands seem to support this assumption.

5. We have seen that the metamorphosis of the skin of *Amblystoma punctatum* consists in the development of the yellow spots of the adult. What has been said in the foregoing pages about metamorphosis means then, that the mechanism which underlies the development of the yellow spots contains one factor which does not belong primarily to the specific properties of the skin, but is formed at some place inside of the body of the organism, at a certain time, and is carried into the skin secondarily. This factor is not specific even for the individual within the species; it is produced by every individual in the same way and therefore can be replaced for the skin of one individual by the body of any other individual of this species, without changing the final result. Furthermore: this factor is not specific even for the species as it can be produced by another species also, as the heteroplastic grafts have shown. Hence one of the most prominent characteristics of this factor is its non-specificity, it is neither specific for the organ nor for the individual nor for the species.

In addition to this non-specific factor a second factor apparently has manifested itself in the mechanism of the development of the

⁸ T. F. Gudernatsch, 1916, p. 358.

yellow spots—it is the factor responsible for the type in which the yellow spots will develop. This factor seems to be highly specific, as suggested by the constancy with which a certain individual and even a certain piece of skin of this individual will develop a particular type of spot, and apparently lies in the skin itself.

As pointed out above, the character of the yellow spots varies greatly, and hardly two individuals can be found in which the spots are alike. The spots vary in (1) the shade of the yellow pigment, (2) the density of the yellow pigment, (3) the size of spot and (4) the number of spots. As a rule in one individual all four characteristics are developed in the same direction; thus, almost uniformly black animals are produced at one end of the scale, while very bright, almost orange yellow spotted animals are found at the other end.

If it were true that this second factor belonged to the specific properties of the skin and was specific for the individual, we should expect that a piece of skin grafted from an individual with the 'almost orange yellow' type of spots, to an 'almost black' animal would nevertheless develop on the 'almost black' host, 'almost orange yellow' spots, for the 'almost orange yellow' type of the factor should have been carried within the graft to the new host. That such is actually the case is shown by some of the experiments of series XXV. It is particularly well expressed in experiments 11-12, and 21-22. Since in each pair both grafts were taken from the same individual we should expect that if the spot developed by one graft is 'almost orange yellow,' the spot of the other graft—if this graft developed a spot at all—should be 'almost orange yellow' too; and this is what actually happened in these two pairs. A similar result was obtained in experiment 33 and experiment 45.

In a similar way must we look upon experiments 13, 28, 45, 11-12 in regard to the behavior of the hosts. In these experiments the hosts were of the black variety. This variety not only is almost bare of yellow pigment, but develops the few faint spots much later—if at all—than the majority of the individuals of this species, and at a time when metamorphosis is almost complete. In these individuals the appearance of the yellow spots cannot be said to indicate that the metamorphosis of the skin has started since the spots appear when it is almost finished, in regard to the rest of the adult charac-

teristics of the skin. If to such an individual a normal piece of skin is grafted it will react in a normal way to the action of the metamorphosis factor and the spots will appear on it; while the host will not react in a normal way and no spots will appear, or only much later if at all, since the second factor necessary for the development of the yellow spots, being a specific factor contained in the skin itself, is either absent from the skin of this individual, or if present is of such a specific type as to permit development of the spots only to a certain decreased amount and after a long time. This result was actually obtained in the group of experiments under discussion.

Quite similar results were furnished by the experiments of Weigl and several other investigators who studied the behavior of the transplanted skin of Amphibians.

Comparing this second factor with the first one, we see that its chief characteristic is an apparent specificity. It is specific for the organ itself, because it cannot be furnished to it by the rest of the body, and it is specific for the individual because it cannot be replaced by the corresponding factor of another individual.

It thus appears that two factors, easily distinguishable, have been revealed as involved in the process of the development of the yellow spots. One of them is a non-specific factor, the other a specific one. The first one, it seems, may be identical with a chemical substance such as a hormone; the nature of the second one is entirely unknown, but in common terms it might be called a 'specific structure.'

I should not have expounded these facts at such length if I did not think them particularly apt to throw some definite light upon the meaning of what we call 'specificity.'

From the experiments reported here, we might well form the opinion that each individual of *Amblystoma punctatum* carries in its skin a specific structure, which is predetermined and according to which the yellow spots of one individual must develop certain definite characteristics different from those of other individuals in which the corresponding predetermined specific structure was different. This structure is unchangeable as is also the type of the spots which develops from this structure; and this is what is ordinarily termed 'specificity.'

The following instance however, shows that these conclusions

would have been premature. In the course of certain experiments not yet published, a series of *A. punctatum* larvae were fed on thymus gland and controlled by another series kept on normal diet (earthworms). Both series were kept in daylight and at relatively high temperatures—up to 30°C. Many of the animals metamorphosed and developed the yellow spots. All individuals of the thymus-set, without exception showed entirely orange yellow, extremely dense spots, no matter how large or how numerous the spots were. Among the worm-fed animals there was a wider variation in regard to the color and density of the spots—from a relatively light yellow and dense type to a very intense yellow ('almost orange yellow') and very dense type; but there was not one individual whose spots were nearly like those of the thymus-animals, (i.e., nearly so reddish yellow and so dense), and this in spite of the fact that all individuals of both sets were the offsprings of one female. The difference in the color was so striking that anyone could have picked out without hesitation the thymus-animals had they been mixed up with the worm-animals. Hence there is a means to change that structure which we thought—from our grafting experiments—to be specific.

From this it seems to me that we cannot refer specificity to an individual or to a certain group of individuals, but that we must refer specificity of a certain type to the methods which we employ in trying to change the type. If our method is unfit to change it, the type appears to be specific; if our method is able to change it, the type seems non-specific. Of course, in this way, we should agree that specificity is only relative and classifying, but not absolute and not real.

V. SUMMARY.

1. From a larva of *Amblystoma punctatum*, each of the two halves of the skin of the head, including one eye, was grafted to another larva of the same species. The animal, from which the grafts were taken, was killed; the two animals to which the pieces of skin had been grafted, constituted one experimental pair.

2. In each pair, the appearance of the color characteristics of the adult *Amblystoma punctatum* skin (network and yellow spots) in the two hosts and in the two grafts, was recorded and these dates were compared with each other.

3. The result was:

a. The skin grafts developed the network and the yellow spots not simultaneously with each other as they would have done if left with the animal from which they were taken, but simultaneously with the host; they metamorphosed only if the host did.

b. This resulted in the establishment of a more or less considerable difference in the time of development of the adult characteristics between the two pieces of skin, provided such a difference existed between the respective hosts. The difference ranged from seven days to five months with regard to the network development, and from three days to four months with regard to the development of the yellow spots. Metamorphosis of the skin graft was retarded or accelerated by the host.

c. The grafted eyes showed a similar time relation with regard to the breaking up of the yellow ring.

4. Pieces of skin and eyes, which were grafted from larvae of *Amblystoma punctatum* to larvae of *Amblystoma tigrinum*, not only metamorphosed also on this host of a foreign species, but showed, with regard to metamorphosis, the same time relation as on a host of the same species.

5. Individual types of the yellow spots of the skin of one individual remained unchanged even when the skin was grafted to an individual whose skin developed yellow spots of another type.

6. From these facts it is evident that at least two factors are involved in the mechanism of the development of the yellow spots of *Amblystoma punctatum*:

a. One factor, which is responsible for the kind of yellow spots that will develop, and which is contained in the skin itself;

b. Another factor which may be called 'metamorphosis factor' and which may be characterized as follows:

1. It is necessary to start the process of the development of the yellow spots; i.e. metamorphosis of the skin;

2. It is not contained in the skin; but produced by the body or some particular organ of the organism;

3. It is non-specific;

4. It may be identical with an agent like thyroid-substance.

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PLATE 1

EXPLANATION OF FIGURES

1 Larva of *A. punctatum*, kept at a low temperature (about 15°C. average) in a dark room and fed on worms. It shows the first color stage (even yellow brown). Cold worms, experiment 7; hatched on May 3, 1916; land-condition December 12. Photographed from live specimen October 25, natural size. (Photo 16.)

2 Larva of *A. punctatum* kept at a low temperature (about 15°C. average) in a dark room. It shows the second color stage ('Network') at an advanced stage. Cold worms, experiment 8; hatched on May 3, 1916; land-condition November 14. Photographed from live specimen on October 25; natural size. (Photo 14.)

3 Larva of *A. punctatum* kept at a high temperature (about 25°C. average) in a light room and fed on thymus. It shows the beginning of the third color stage (separation of the network into yellow spots). The animal had 102 yellow spots when photographed. Warm thymus experiment 2; hatched on May 3, 1916; land-condition October 19. Photographed October 9 from live specimen; natural size. (Photo 2.)

4 Larva of *A. punctatum* kept at a high temperature (about 25°C. average) in a light room and fed on worms. It shows the third color stage ('Separation of Network') in an advanced stage. The yellow spots are mostly faint and the surface of the skin appears dull. Warm worms, experiment 2; hatched May 3, 1916; and-condition September 29. Photographed from live specimen October 10; natural size. (Photo 5.)

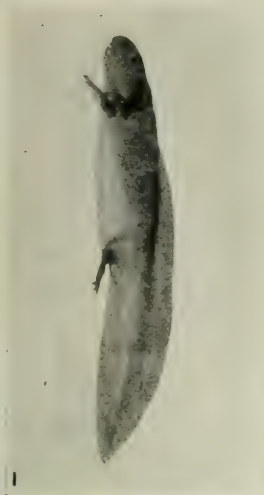


PLATE 2

EXPLANATION OF FIGURES

5 Eye of *A. Punctatum* showing the iris divided into two sections, in a very early larval stage (about two weeks after hatching). The inner section is pure yellow and does not contain melanophores. Magnification (?).

6 Eye of *A. punctatum* about 2 months after the animal was set on land condition. The iris appears dark brown and contains only a few yellow chromatophores. Magnification (?).

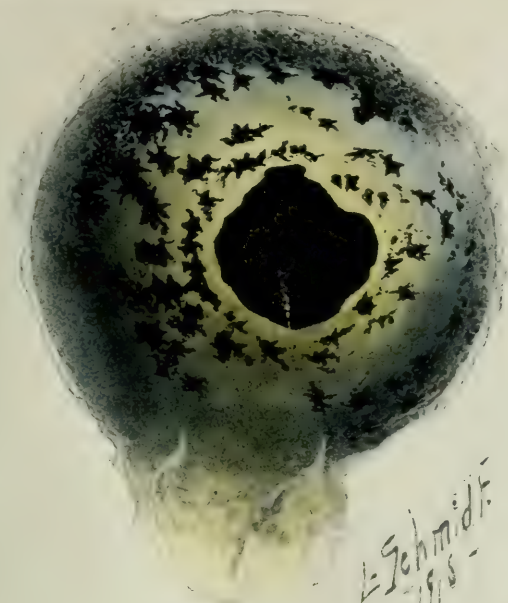


PLATE 3

EXPLANATION OF FIGURES

7-12 Schematic figures to illustrate size, color, density, number and distribution of the yellow spots. Printed sketches of a uniform size, showing the outlines of the animal's body, were used and the spots were drawn not in their actual size but enlarged in proportion to the size of the sketch. Spots of 'almost orange yellow' color are shown orange, while 'light yellow' spots are represented by yellow. Dotted spots are indicated by black dots on the yellow color. The borders of the graft, where they still were visible, are indicated by a white line.

7 Series XXV, experiment 21. Spot 1 is the reddish yellow spot of the graft; the spots of the host were light yellow. Drawn on December 14, 1916, from live specimen.

8 Series XXV, experiment 22. The spots of the host are light yellow; spots, 1, 2 and 3 are very bright reddish yellow spots. Spot 1 and 2 are spots of the graft; the history of spot 3 has not been recorded, but it probably also belongs to the graft (see text p. 258). Drawn on December 14, 1916, from live specimen.

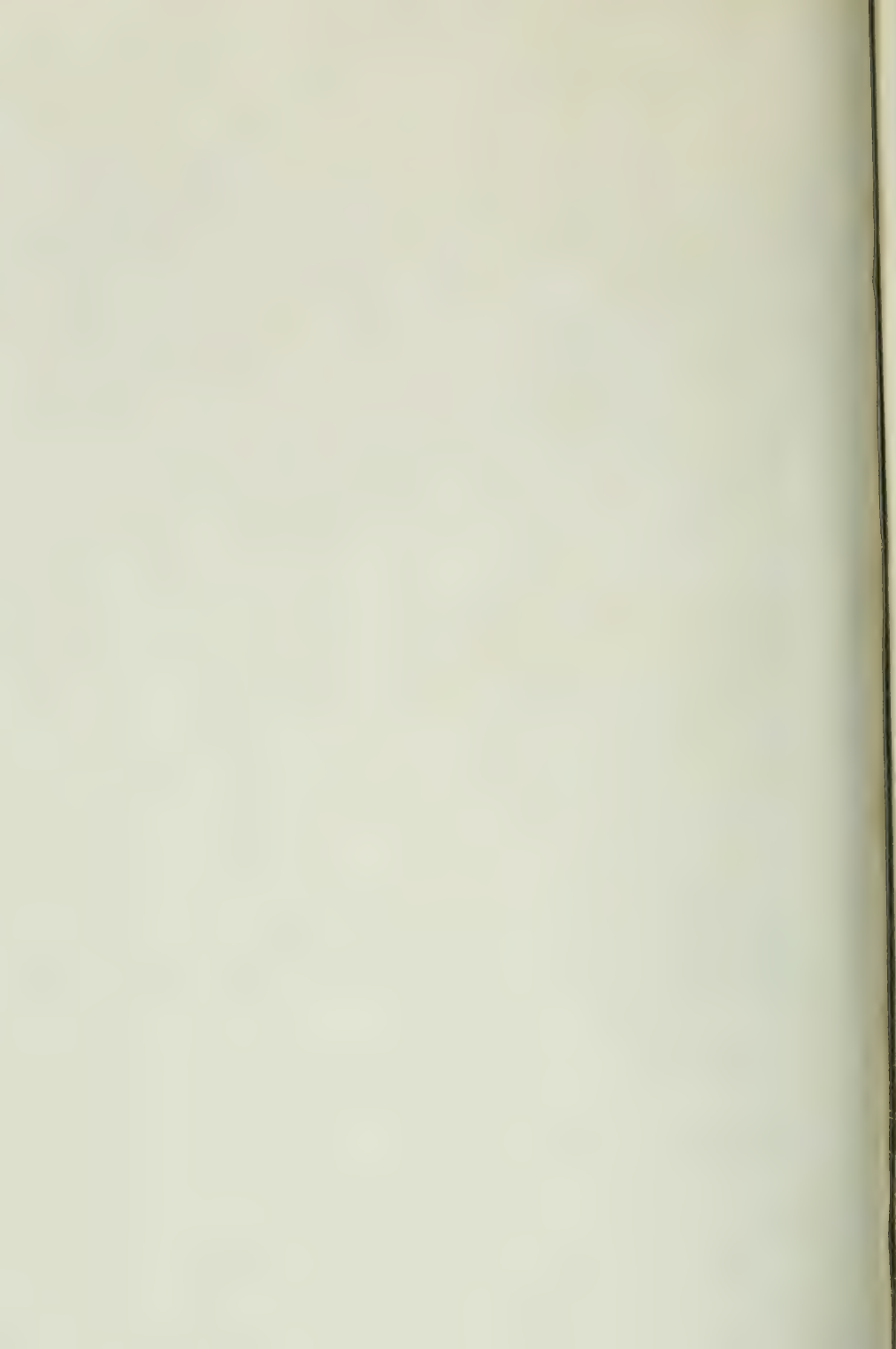
9 Series XXV, experiment 45. The spots of the host are either pale yellow (tail) or they are faint dotted spots (body); spot 1, which belongs to the graft, is bright and almost orange yellow. Drawn December 24, 1916, from live specimen.

10 Series XXV, experiment 28. The host developed on its body only a few light yellow and mostly small spots while the largest part of the body is without spots. Drawn December 18, 1916, from live specimen.

11 Series XXV, experiment 11. The host developed only small and faint dotted yellow spots, while the graft developed a relatively large, very bright, almost orange yellow spot (spot 1). Drawn December 13, 1916, from live specimen.

12 Series XXV, experiment 12. The host developed only 1 faint dotted yellow spot on the whole body; the graft developed 4 spots, 2 of them (spot 1 and 2) were bright and almost orange yellow. Drawn December 13, 1916, from live animal.





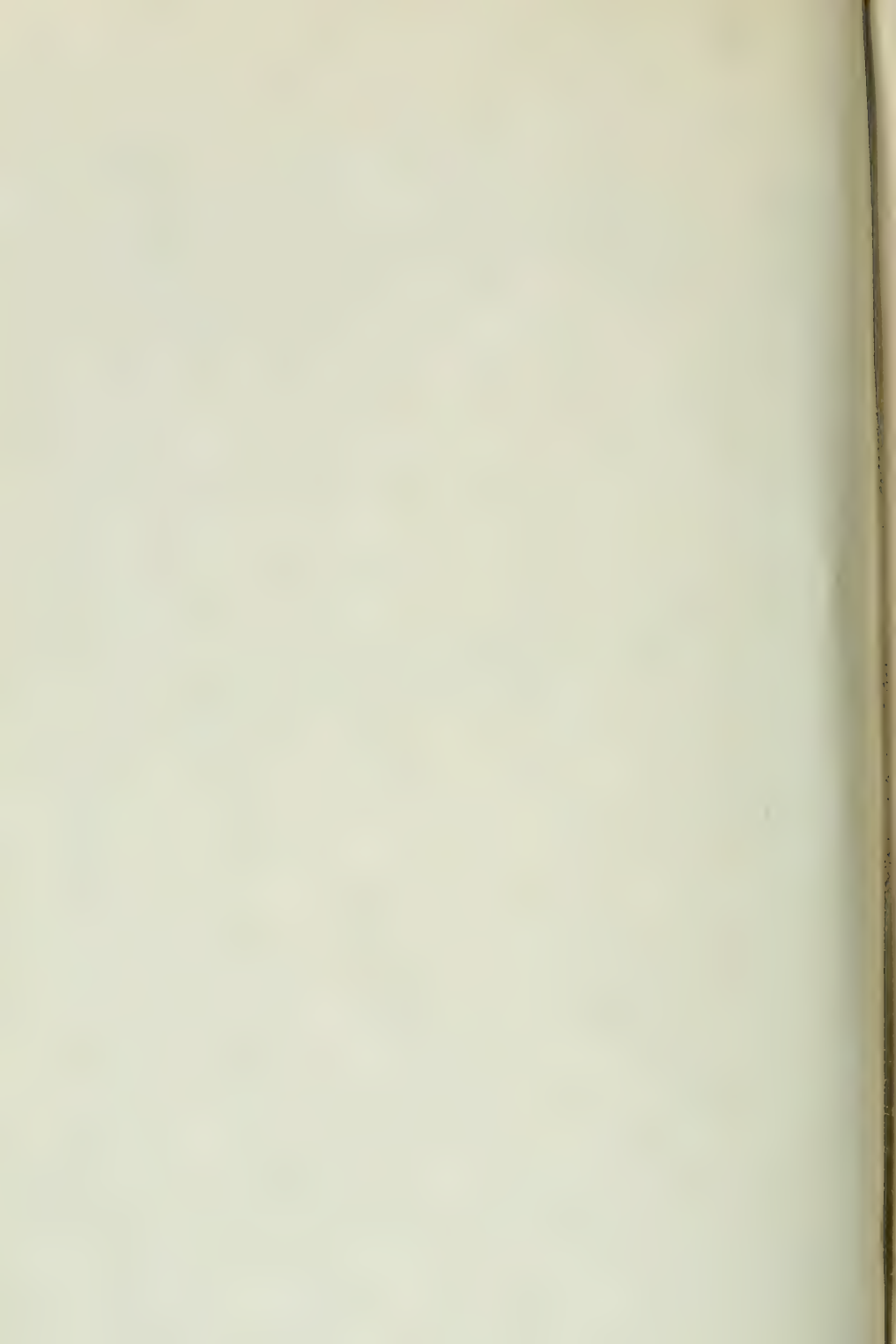


14



16





THE THERAPEUTIC VALUE OF ORAL RHYTHMIC INSUFFLATION OF OXYGEN.

WITH DESCRIPTION OF A SIMPLE APPARATUS FOR ITS EXECUTION.

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The literature on the relation of oxygen to the processes of life, the rôle which oxygen plays in internal and external respiration, and the availability of that gas as a therapeutic measure, is immense. I shall not attempt to review it even in a cursory way, but it may not be amiss to discuss some of the problems of this subject, especially those which we encounter in recent medical literature. We may conveniently divide the discussion of our subject into two aspects: the physiologic and the therapeutic.

The Physiologic Aspect.

It seems to be the dominant opinion among physiologists that inhalation of pure oxygen or air strongly enriched with oxygen is of no greater oxidative value to the normal organism than the inhalation of simple atmospheric air, which contains about 20 per cent. of oxygen. This view seems to be upheld by two considerations. In the first place, it is a fact that the oxygen in the blood is carried chiefly by the hemoglobin, which, it is generally assumed, is nearly saturated with oxygen and would not take up more of it even if the individual were breathing pure oxygen. The only part of the blood which may take up more oxygen when offered under higher pressure is the serum; but it seems to be generally accepted that the amount of oxygen which the serum is thus able to take up is a small and negligible quantity. The second influential consideration is the assumption, which seems now to be quite generally accepted, that the decision as to the amounts of oxygen which the organism is to absorb rests finally

and exclusively with the body cells, that is, with their necessities, activities and storing capacities, and is not influenced by the oxygen pressure within the tissue lymph. In other words, oxidation, the chief process of life, depends on cell activity and not on the tension of oxygen within the blood or within the tissue lymph.

The question under discussion has been the subject of numerous investigations. Lavoisier and Seguin, as far back as 1789, were the first to draw the conclusion from their experiments that "there was no increase in the vital processes as the result of breathing pure oxygen." Probably the last, and surely one of the best and apparently most careful investigations, is the one that was published a few years ago in this country by Benedict and Higgins.¹ They say that a critical examination of the entire literature strongly favors the belief expressed by Lavoisier. But they say that "nevertheless we find that there is much that is lacking in the evidence thus far secured, so that the matter is not yet definitely settled." Their careful investigations, however, led them to a similar result, namely, that there is no difference in the gaseous metabolism between breathing ordinary air and breathing oxygen mixtures of 40, 60 or 90 per cent.

But even with regard to this careful research, Krogh² said: "It is extremely probable, however, that there is in these experiments a slight systematic error," and he comes to the conclusion that the "breathing of oxygen does increase the metabolism to some slight extent."

Apparently there are many factors of greater or lesser importance that have to be taken into consideration before we shall be justified in accepting as final any estimate of the extent of the influence that the inhalation of air with a higher partial pressure of oxygen may exert on the normal gaseous metabolism. The hemoglobin is even normally not completely saturated with oxygen, nor is the oxygen in solution in the blood serum normally an entirely negligible quantity, and it is certainly not negligible during an increase of the partial pressure of oxygen of the inhaled air.

1. Benedict and Higgins: Effects on Men at Rest of Breathing Oxygen-Rich Gas Mixtures, *Am. Jour. Physiol.*, 1911, 28, 1.

2. Krogh, August: The Respiratory Exchange in Animals and Man, London, Longmans, Green & Co., 1911.

There is no real quantitative analysis of the amount of oxygen present in the tissue fluid. The analysis of the various body secretions does not mean the same as an analysis of the tissue fluid. There are some authoritative investigators who still believe in the storage of oxygen, intramolecularly or extramolecularly, in loose chemical combination or in physical solution. There are other considerations to be solved; but I shall not attempt to discuss any of these points. However, I wish to call attention to one factor which has not yet been taken properly into account, or at least not from the proper point of view, as it seems to me, and that is that element in biology which I designated, a decade ago, as the factor of safety.

All experiments under consideration start implicitly from the point of view that the oxygen content in the air is the physiologic amount sufficient for the maintenance of the life of the animal under normal conditions. But is this entire amount also indispensable? It has been established by many experiments that the organism remains in a normal condition even if the partial pressure of the oxygen in the air amounts to only 12 or 13 per cent. What is the significance of the considerable surplus of oxygen that the animal is normally breathing? From my point of view it presents a factor of safety in the function of respiration, a factor which I have shown to exist in most of the functions of the animal body.³ I shall not attempt to discuss again this problem in its details. But it will not be amiss to give an example taken from every day life which will suffice to illustrate the meaning of the underlying principle of the factors of safety and the simplicity of its requirements. Let us take, for instance, two houses of about the same size, of which we have good reasons to expect that each house uses up the same amount of coal in a given time. But while the occupant of one house, who may possess larger means and a greater foresight, lays in a larger supply of coal, let us say, twenty tons at a time, we find the occupant of the other house, perhaps because of lack of means or the necessary foresight, provides himself with only one ton of coal at a time. The difference in the fate between the occupants of the two houses becomes

3. Meltzer, S. J.: The Factors of Safety in Animal Structure and Animal Economy, *THE JOURNAL A. M. A.*, Feb. 2, 1907, p. 655.

strikingly evident when unexpectedly necessities arise for greater consumption of coal, and when, furthermore, these necessities set in under circumstances of time and place that make coal difficult to obtain.

Life is characterized by the instability and variability of its states, and all its functions must therefore be so arranged as to be capable of being ready to meet the highest possible demands. The function of respiration has for its object chiefly the provision of the organism with oxygen, properly and safely. While, as far as the muscle system is concerned, the provision of the respiratory function with factors of safety is under normal conditions surely of a satisfactory extent, because, as we know, the muscle is capable of continuing its contractions even if the oxygen tension of its surrounding atmosphere is fairly low, it is different with the central nervous system. Here we know that a comparatively slight reduction in the oxygen tension interferes profoundly with the activities of this system. Are we justified in assuming that the addition of 7 or 8 per cent. of oxygen is just the limit of the factors of safety which the respiratory function may need and use under all conditions? Furthermore, in breathing normal atmospheric air, what is the fate of the 7 or 8 per cent. of oxygen present in the normal air above the amount actually indispensable for maintenance of life? Is this oxygen stored away somewhere and in some manner, to be ready for immediate use when needed, or is it normally unabsorbed and got rid of in the same manner as is assumed to be the case when 20 or 30 per cent. of oxygen is artificially added to the atmospheric air?

The Therapeutic Aspect.

The extensive experiments on which the prevailing view has been founded, namely, that the addition of oxygen to the atmospheric air does not affect the normal metabolism, were made on normal resting individuals. But the processes of life, even in the normal individual, and even while at rest, cannot be represented by a straight line. The processes vary continuously within certain limits; that is life. These variations in the demands have to be met by variations in the supplies which have to be ready in store to the extent corresponding

to the maximum limits of the demands of normal life. The 7 or 8 per cent. surplus of oxygen in the atmospheric air is apparently sufficient to meet the maximum demands of the respiratory function of the normal individual when at rest. But would it be sufficient to cover the demands when the individual is engaged in hard work? We know that unusual physical effort leads to shortness of breath, to dyspnea. Could this not be remedied by adding oxygen to the atmospheric air which the hardworking person has to respire? This is indeed the case, a fact which has been discovered and established by Leonard Hill⁴ and his co-workers. Hill and Flack⁴ found that inhalation of oxygen makes running up and down stairs easier, can be accomplished in a shorter time, relieves the feeling of dyspnea, and restores strikingly the vigor after fatigue from boxing, etc. They believe that the fatigue which follows an athletic feat is cardiac in origin and due to want of oxygen. Haldane,⁵ whose great merits in the elucidation of the physiologic relations of carbon dioxide to the function of respiration are well known, was inclined to explain Hill's observation as a result of the deep breathing which is apt to occur in persons to whom oxygen is administered. Haldane's view was later refuted by new experiments, carried out by Hill and Mackenzie,⁶ which brought new evidence that the favorable effects that they have obtained were indeed due to the inhalation of oxygen.

From the observations of Hill we learn that the addition of oxygen to atmospheric air exerts a favorable influence under conditions of greater bodily activities. Is inhalation of air enriched by oxygen also capable of exerting a favorable influence when the body is in a condition of rest but is undergoing pathologic processes? In other words, is there any evidence that inhalation of oxygen is capable of exerting a therapeutic effect? This question concerns a wider field than I intend to cover in this paper. There is no doubt now as to the therapeutic value of inhalation of air-oxygen mixtures in cases of poisoning

4. Hill and Flack: The Influence of Oxygen on Athletes, 1909, 38, *Jour. Physiol.*, 28.

5. Haldane, J. S.: The Effects of Previous Forced Breathing and Oxygen Inhalation on the Distress Caused by Muscular Work, *Jour. Physiol.*, 1909-1910, 39, *Proc. Physiol. Soc.*, London, 1.

6. Hill and Mackenzie: *Jour. Physiol.*, 1909-1910, 39, *Proc. Physiol. Soc.*, 33.

by gases that affect the normal condition of the hemoglobin. But I wish to confine my remarks to the use of oxygen in certain types of diseases. Here we encounter a very unsatisfactory situation. On one hand, hardly a patient, sick with a respiratory or cardiac disease, will be permitted by the physician in attendance to die without trying oxygen inhalation. On the other hand, not many practitioners will readily admit that oxygen is doing much good in these cases.

Two factors are at the bottom of the skeptical attitude of physicians toward the value of oxygen as a therapeutic agent. In the first place, many clinicians are influenced by the view entertained by the great majority of physiologists that the addition of oxygen to the inhaled air is incapable of exerting a physiologic influence, that it does not affect the metabolism, and that the added oxygen returns unabsorbed. Furthermore, many clinicians and pharmacologists are still under the influence of the teaching that there is no fundamental difference between physiologic and pathologic processes. In this connection, the present attitude of the physiologist Haldane is very instructive. As stated above, Haldane doubted that the favorable influence which, according to Hill, the administration of oxygen exerts on the work of athletes is due to oxygen. Very recently, however, Haldane⁷ came out strongly for the administration of oxygen in inflammatory conditions of the lungs. He says:

It may be argued that such measures as the administration of oxygen are at the best only palliative and of no use, since they do not remove the cause of the pathological conditions. As a physiologist, I cannot for a moment agree with this reasoning. The living body is no machine, but constantly tending to maintain or to revert to the normal, and the respite afforded by such measures as the temporary administration of oxygen is not wasted, but utilized for recuperation.

The second, and probably the more influential factor, is the actual fact that practitioners rarely see any favorable effect that may have been brought about by the administration of oxygen. But the general conclusion drawn from this fact, namely, that the inhalation of oxygen is incapable of exerting a favorable influence in pathologic conditions, is unjustifiable. The failure to see such an influence is probably due essentially to the inefficient method of administration. In

7. Haldane: Brit. Med. Jour., Feb. 10, 1917. Benedict and Higgins (Note 1).

most instances oxygen is given in a weak current through a funnel which is kept an inch or more from the mouth of the patient. Under these conditions we can hardly speak of "inhalation of oxygen." The atmospheric air which the patient actually inhales is probably not richer by more than 2 per cent. of oxygen, if by so much. On the other hand, the funnel may be instrumental in making the inspired air richer in carbon dioxid and surely makes the air over the face warmer, an unpleasant sensation to the patient, who prefers to be fanned and cooled off. Therefore, we often see the patient pushing the funnel away or turning the head away from it.

The Author's Apparatus and Experience in the Use of It.

My personal experience dates back about two years. I was called up one night by a very unhappy mother who told me that her only son was dying and begged me to come over. The athletic young man of 25 years was sick with pneumonia, Type II. I arrived at about 2 a.m. and found several physicians around the patient, who was unconscious and deeply cyanotic. Respiration was rapid and very shallow. He had no corneal reflex and there was a thready pulse of about 190 per minute. The patient was apparently dying and the physicians expected death to take place within the next ten or fifteen minutes. Nurses were administering oxygen in the usual manner, that is, through a paper funnel, kept at some distance, the gas bubbling through a wash bottle at a moderate pace. I disconnected the rubber tube from the foot bellows of my pharyngeal insufflation apparatus⁸ that I brought along with me, and connected it with an oxygen cylinder. I then inserted the pharyngeal tube in the mouth (not in the pharynx), turned on the oxygen and started working the respiratory valve. Within a short time after the beginning of the oxygen insufflation in the new manner, the cyanosis disappeared and the face of the patient became actually pink. Several minutes after the discontinuance of the insufflation, the cyanosis began to

8. Meltzer, S. J.: History and Analysis of Methods of Resuscitation, Med. Rec., New York, July 7, 1917; Pharyngeal Insufflation, a Simple Method of Artificial Respiration, THE JOURNAL A. M. A., May 11, 1912, p. 1413; Simple Devices for Effective Artificial Respiration in Emergencies, *ibid.*, May 10, 1913, p. 1407.

reappear gradually. In the course of the next five hours the experiment was repeated numerous times and invariably with the same result, that is, the cyanosis disappeared promptly during the insufflation of oxygen and reappeared several minutes after cessation of the insufflation. There was no perceptibly favorable influence on the other symptoms of the patient, except perhaps on the pulse. In the morning hours it came down to 180 per minute and was perhaps slightly fuller. At any rate, in the bedside parlance, it could be honestly stated that "the patient was holding his own." The waste of the gas was considerable. A tank of oxygen was used up in a short time. About 7:30 in the morning the supply of oxygen of the neighboring drug stores became exhausted, and it took some time before another tank of oxygen could be procured. Five minutes after the last insufflation had to be discontinued the heart stopped suddenly. The resumption of the insufflation of oxygen about ten minutes later had no effect whatever.

This patient was not saved; but nobody could have expected it when beginning treatment at that terminal stage. His life, however, seemed to have been prolonged, and it is impossible to state how much longer death would have been deferred, if the supply of oxygen had not given out. But this is an insignificant issue compared with the fact that the insufflation exerted an unmistakable effect on the profound cyanosis. Cyanosis, of whatever origin and nature, is a sure evidence that the respiratory function is profoundly disturbed. In the present case the cyanosis promptly disappeared on the administration of oxygen by the new method. Although we are dealing with only one case, each instance in this case in which the cyanosis disappeared on the insufflation of oxygen and reappeared on stopping the insufflation presents a complete experiment, an experiment that we were able to repeat at will. We made more than a dozen such experiments, and the results were invariably the same. These experiments permit at least the provisional conclusion that the insufflation of oxygen by the method employed in this case affects favorably the function of respiration when it is pathologically profoundly disturbed.

In the case here mentioned the apparatus employed was provisionally arranged and applied in great haste without consideration for

the great waste of oxygen and other shortcomings. After observing the results, which seemed to be very encouraging, I devised a special apparatus for our purpose which is simple, inexpensive and works very satisfactorily.

The thick walled rubber tubing that is connected with an oxygen tank terminates in a strong rubber bag which, at its distal end, is connected with the proximal end of the respiratory valve.⁹ The distal end of the last named device is connected by means of a short piece of rubber tubing with a flat metal tube which we may designate as a "hollow tongue depressor." If the ring of the respiratory valve is kept in the inspiratory position (right side), when the oxygen is turned on, the gas streams through the tube into the bag and from there escapes through the respiratory valve and the tongue depressor. If the ring is kept in the inspiratory position (left side) the oxygen cannot pass through the respiratory valve, and accumulates within the bag. The accompanying illustration shows the last mentioned position.

The tongue depressor should be inserted in the mouth not much farther than the middle of the tongue, so that, if the patient is conscious, the presence of the depressor may cause no gagging or other discomforts. The lips should be kept closed. The ring should be moved from left to right and from right to left (a respiratory circle) about twelve times per minute. The oxygen should be turned on slowly, and the velocity of its escape should be controlled, so that it does not cause an overdilatation of the bag during the expiratory pause. The turning of the ring to the right should be done slowly, so that the inspiration may develop gradually; the turning to the left is preferably done abruptly. The expiratory air escapes during the closure of the valve through the nose and through the aperture that appears above the closed valve when the ring occupies an expiratory position. It is advisable to time the inspiratory insufflation synchronously with the inspirations of the patient. However, I found that after a while the respiratory phases of the patient become involuntarily adapted to the phases of the insufflation.

9. A description of this device is contained in the Medical Record, New York, July 7, 1917.

I have tested on myself the action of insufflation of oxygen by means of this apparatus. When the insufflation is carried on under moderate pressure, there are no unpleasant sensations whatever. When it is done under too much pressure, the surplus of oxygen escapes through the nose and never enters the esophagus; but it causes some unpleasant sensations which conscious patients will probably not be willing to stand for any length of time.

My chest was examined by auscultation while I was receiving oxygen insufflation. It was found that each insufflation produced a distinct inspiratory blowing sound which was distinctly recognized even while I was holding my breath. It seemed, further, that the oxygen was capable of entering my lungs even when I was endeavoring to keep the glottis in a state of adduction. Some other objective and subjective effects of the insufflation experiments on myself will be mentioned later.

The results of the observations made by the method of auscultation induced me to make a few experiments on animals. In deeply anesthetized cats, with the thorax split transversely, the insufflation by means of the apparatus was sufficient to distend the lungs moderately with each insufflation, even when the "tongue depressor" was inserted in the mouth, as in the human being, not farther than the middle of the tongue. It appeared, however, that when the tongue depressor was kept at that position, life could not be kept up indefinitely. When the tongue depressor was inserted in the pharynx, the oxygen entered readily the gastrointestinal canal, which had to be prevented by keeping the abdomen compressed. With this precaution the artificial respiration was excellent and the animals remained in good condition. But in this arrangement the method is identical with the method of intrapharyngeal insufflation that I have described elsewhere, and is not applicable to conscious patients.

Dr. A. L. Meyer of our laboratory performed on himself a couple of experiments with the apparatus. After having been insufflated for about eight minutes, he analyzed his expiratory air at the end of the insufflations and found it to consist of nearly pure oxygen. The nitrogen of the atmospheric air was displaced by the oxygen.

The foregoing observations demonstrate that by using the "apparatus for oral insufflation of oxygen" under moderate pressure, oxy-

gen undoubtedly enters the lungs, assists in distending them during inspiration, and displaces largely the nitrogen of the "dead space." The assistance that it renders to the inspiration ought to be of special value when the respiration of the patient is very shallow. The rhythmic character of the insufflation is, on the other hand, of value, as it does away with the resistance which the pressure of the continuous insufflation undoubtedly offers to the expiration, especially when, owing to the low vitality of the patient, the expiratory efforts fail to get succor from the activity of the expiratory muscles. Haldane administers the oxygen by means of a mask. The interruption of the continuous stream of oxygen in his method depends on the force of



The oral insufflation apparatus. The rubber tubing which is connected with an oxygen tank terminates at its distal end in a strong rubber bag, which in turn is connected with the "respiratory valve." The ring of this device occupies in this illustration an expiratory position; hence the distention of the bag. Above the ring an aperture can be seen, which appears only when the valve is closed (expiratory position). The respiratory valve is connected at its distal end by means of a short piece of rubber tubing with the hollow "tongue depressor." The T tube is here unessential.

the expiration, which is expected to close a mica valve and, at the same time, prevents the entrance of the expiratory air into the bag that stores up the oxygen. I developed my simple apparatus about eighteen months previous to the appearance of the communication of Haldane. I then gave up the use of the mask even in my pharyn-

geal apparatus, mainly because it is liable to drive some deleterious material that may be present in the nasal and the postnasal cavities into the trachea and the lungs, there causing an infectious inflammation. Hill and Mackenzie⁶ stated that in giving oxygen during athletic work they did not use the mask, because it increases the dead space. In dealing with sick persons some other points must be regarded as more important. Either the patient is already unconscious, in which case the respiration will be shallow and the expiratory force will be insufficient to close the valves and overcome the pressure within the bag. Or the patient is still conscious and he will surely feel the face piece, which fits over mouth and nose and is kept in position by an elastic strap, as a great inconvenience, and will not tolerate it. Haldane says he had but few opportunities to give his method a practical trial, but he reports that in a case of valvular disease he has seen the cyanosis "clear up at once on the administration of oxygen." There is some other difference between Haldane's method and mine. By Haldane's arrangement the oxygen is accumulated in a bag of thin vulcanized rubber. This oxygen enters, then, through mouth and nose, under comparatively low pressure, and it was not established whether the stream enters the lungs with any force capable of causing any degree of artificial inspiration. The bag in our apparatus, on the other hand, has thick walls and drives out the oxygen under a pressure which is sufficient, as we have previously shown, to cause a deeper inspiration and as a consequence also a stronger expiration. Haldane says that he knows from experiments on himself and others that the immediate effect of suddenly giving an abundance of oxygen may sometimes be unpleasant. I have taken oxygen many times by means of my apparatus and sometimes as long as eighty minutes, and never felt any unpleasantness from it. Under stronger pressure and prolonged insufflation there might arise a sensation of dryness. This can be easily remedied by interpolating between the oxygen tank and the bag a wash bottle containing a Ringer solution. I agree, however, with Haldane that the oxygen should be turned on slowly.

Not being any longer in private and hospital practice, I am not in the favorable situation to have many opportunities for testing personally the value of the method. I am, nevertheless, in a posi-

tion to report encouraging and instructive results obtained in a few cases, three of which were observed at the Rockefeller Hospital. I have to thank Dr. Chickering for the details in these cases. The first case was a most gratifying one. One forenoon in November, 1916, Dr. Cole, the director of the hospital, telephoned to our laboratory asking me to come over and administer oxygen with my apparatus to a pneumonia patient. To my reply that I would be over in half an hour, as I had to finish an experiment, Dr. Cole remarked that in all probability it would then be too late. I went over immediately and started the administration of oxygen by means of the oral insufflation. That patient recovered. Concerning the details of that case I shall quote Dr. Chickering.

Report of Cases.

Case 1.—The patient came in on the second day of his disease having signs of consolidation of both lower lobes of the lungs. . . . A Type II pneumococcus was recovered from the sputum . . . He was treated with ethyl-hydrocuprein (optochin) on the third day of his disease, and this treatment was continued during the fourth, fifth, sixth and seventh days . . . partly by mouth and partly intramuscularly. On the fourth day of his disease the patient appeared extremely ill, temperature being 103.4 F., pulse 152, and respiration 40. He was delirious, color was ashy gray, lips and finger tips very cyanotic and tracheal râles were marked. At 10.45 a. m. the patient was given oxygen according to Dr. Meltzer's method intermittently for about two hours. While the oxygen was being given, his color changed surprisingly; his lips and ears lost their cyanotic color and took on a pinkish tint, and his general condition seemed decidedly improved. The following morning the patient's temperature and pulse were lower, 100.5 F., pulse 104 and the respirations 32. His temperature gradually came down by lysis to normal on the twelfth day of his disease. Convalescence was uninterrupted, no complications developing except a moderate amount of sterile pleural effusion which gradually cleared up without aspiration.

Case 2.—The patient was admitted, Feb. 19, 1917, and the oxygen treatment was begun on the twelfth day of the disease, one hour before death, February 28. The patient received ethyl-hydrocuprein treatment every day from the fourth day of his disease to the twelfth, the day of his death. On the day of his death, his blood culture contained innumerable colonies of pneumococci of Type II. "On the afternoon of his death his color suddenly changed . . . and severe cyanosis developed." Oxygen treatment was started. "While the oxygen was being administered, patient's cyanosis became less intense, but as soon as the oxygen was stopped cyanosis rapidly returned."

Case 3.—The patient was admitted to the hospital, Feb. 25, 1917, on the first day of the disease. "The next morning the whole right chest was involved posteriorly, and on the following day signs appeared anteriorly. At the same time the left chest became filled with fine moist râles. From the sputum an atypical Type II pneumococcus was obtained. Blood culture was sterile, blood count 29,000. . . . On the fourth day of the disease the signs became more marked for the left lower lobe; the temperature rose to 104.5 F. and the pulse to 140. The patient became pale and cyanotic. The extremities were cold and expirations labored and moist. Ethyl-hydrocuprein was begun at 2 p. m., and oxygen at 3 p. m.¹⁰ . . . During the afternoon the pulse rate gradually increased to 142 (8 p. m.). The semicomatose, delirious condition deepened into unconsciousness about 7 p. m. From then on, the color could be maintained with oxygen, but the pulse quality became poorer; respirations increased from 44 to 60, and the patient's throat gradually filled with mucus. There was dulness over the left lower lobe, with râles throughout the left chest. From 2 a. m. to 6 a. m. the time of death, it was necessary to use oxygen continually in order to maintain respiration. If the oxygen was stopped for a few minutes respiration became more shallow and slow, and a dull blue flush appeared on face. This immediately disappeared with more oxygen. Gradually her color and respiration failed in spite of continual oxygen and the pulse, which was good almost to the end, because more feeble and gradually less rapid until it failed entirely at 6 a. m."

COMMENT.

In these three cases the pneumonia was due to pneumococcus of Type II, for which an efficient antiserum has not yet been developed. In all three cases the oxygen treatment was instituted very late, when the condition of the patient already appeared to be quite hopeless. Nevertheless in all three cases the oxygen insufflation manifested the tendency to exert a favorable influence. Even in the second case, in which the oxygen treatment began only one hour before death, the cyanosis of the patient became less intense while the oxygen was administered, to return rapidly when the oxygen was discontinued. This favorable effect on the cyanosis was still more striking in the other two cases, in which each short series of insufflations produced a change of color which outlasted the insufflation by shorter or longer periods. In the first case the face had a leaden, ashy gray color which is characteristic for oxygen deficiency.¹¹ This

10. In the first few hours the oxygen was administered without expiratory interruptions.

11. Haldane: Brit. Med. Jour., Feb. 10, 1917.

color disappeared quite soon after I started the insufflation, and the face gradually assumed rather a pinkish appearance. When I first saw the patient I understood why Dr. Cole thought that the administration of oxygen after half an hour might prove to be too late. I had little hope that the administration of oxygen would save the life of the patient. He was unconscious, had no corneal or lid reflex, the pulse was rapid, small, with very little tension, and the tracheal râles had the death sound. Nevertheless, that patient recovered. The third patient, who received oxygen insufflation for about fifteen hours, died; the pneumonia in this case was very extensive; it spread over both lungs. But here again, besides the pronounced action on the cyanosis, there seems no doubt that the oxygen insufflation prolonged the patient's life. We may be, in fact, we ought to be, optimistic enough to assume that in conjunction with the use of some other efficient or even only semiefficient remedies, early insufflation of oxygen may offer a chance even to patients of this type.

The third patient has taught another favorable lesson, namely that the oral insufflation of oxygen may assist in maintaining respiration. In the last few hours of her life, when the insufflation was stopped, the respiration became shallow and slow, and a dull blue flush appeared on her face which disappeared as soon as the oral insufflation was begun again. It acted apparently like artificial respiration. This fact reminds me of the statements previously made with reference to the auscultation of my chest while receiving oral insufflation, and to the behavior of the lungs in animals under the same condition.

I shall record further observations made by Dr. Victor Meltzer on a woman, aged 60, with marked arteriosclerosis and hypertension. For the last two years she was most of the time subject to cardiac dyspnea and had frequent and severe attacks of pulmonary edema. In the last two months of her life, oxygen was administered to her by means of the apparatus for oral insufflation during the attacks of dyspnea as well as those of pulmonary edema. It proved to be of great value, and afforded the patient great relief. When administered at the beginning of an attack of edema, not infrequently it prevented its full development. Among the most noticeable effects were the rapid disappearance of cyanosis.

Before concluding, I shall record briefly two observations made on myself. For experimental purposes I had oxygen administered to me several times by means of oral insufflation for periods lasting between thirty and eighty minutes. Objectively I can state that the insufflation brought high color into my face, which was noticed by persons who did not know of my experiment, and which did not leave me for several hours. Subjectively I found that it removed the sensation of fatigue and that I felt stimulated for the rest of the day. Of course, the latter fact might not have been due to the insufflation of oxygen. Not infrequently a successful experiment may bring about similar results. At any rate, both facts concern the problem of the possibility of a physiologic action of oxygen when administered by rhythmic oral insufflation, a problem which I shall not discuss here.

SUMMARY AND CONCLUSIONS.

In four pathologic cases it was definitely established that the rhythmic oral insufflation of oxygen reduced or removed promptly the cyanosis, and in three of the cases the patient turned even pinkish shortly after the insufflation was begun. Without doubt these prompt effects must be ascribed in the first place directly to the action of oxygen. But it must be admitted that the rhythmic insufflation, since it is capable of assisting in the maintenance of the respiration, may be helpful in the ventilation of the lungs and thus helpful also in the removal of some of the accumulated carbon dioxid. The favorable action of the insufflated oxygen may be explained in various ways. It may simply be due to the presence of the oxygen in the blood serum in greater quantity, as a consequence of the greater oxygen tension in the alveolar air. It may be further assumed that in certain pathologic conditions the hemoglobin is not saturated with oxygen and therefore takes it up readily from the serum, and furthermore, in certain stages of the disease the oxygen thus taken up may be retained by the hemoglobin for shorter or longer periods even after the oxygen tension in the alveolar air and in the serum is again reduced. The same may perhaps be said of the other vital body cells, the nerve cells of the medulla and the cells of the circulatory neuromuscular apparatus. In the course of a disease either the cells are gradually receiving

less and less oxygen, or they gradually lose the capacity for storing up oxygen, or both. There are other possibilities; but I shall not merely speculate as to which of these possible factors were the effective elements in the observations previously mentioned. But I shall point out the following facts. In one case of pneumonia the cyanosis returned immediately when the oxygen insufflation was stopped. In this case surely the vital cells had no longer any storing capacity. This patient died one hour after the insufflation was installed. In the case of another patient the insufflation had at first a marked after-effect when the insufflation was discontinued, but gradually this storing capacity disappeared and the patient was kept alive for several hours longer by continuing the insufflation without intermissions. In this patient, when the insufflation was first begun, some degree of storing capacity of the vital cells for oxygen was still present, but it was on its down-hill course, which the oxygen insufflation could neither revert nor stay. In my first patient each series of insufflations was followed by a period of freedom from cyanosis. These periods were short, but for five hours the periods did not become shorter; "the patient held his own," and he died suddenly when the period before oxygen could be again administered became too long. In this patient the down-hill course was stayed; whether it could have been reverted by further administration of properly timed insufflations of oxygen was at that time more than doubtful. But the condition of the fourth patient, who actually recovered, was, when first seen, as grave as that of the last patient. Nevertheless, the rhythmic oral insufflation of oxygen not only stayed the down-hill course, but undoubtedly assisted in reverting it to normal.

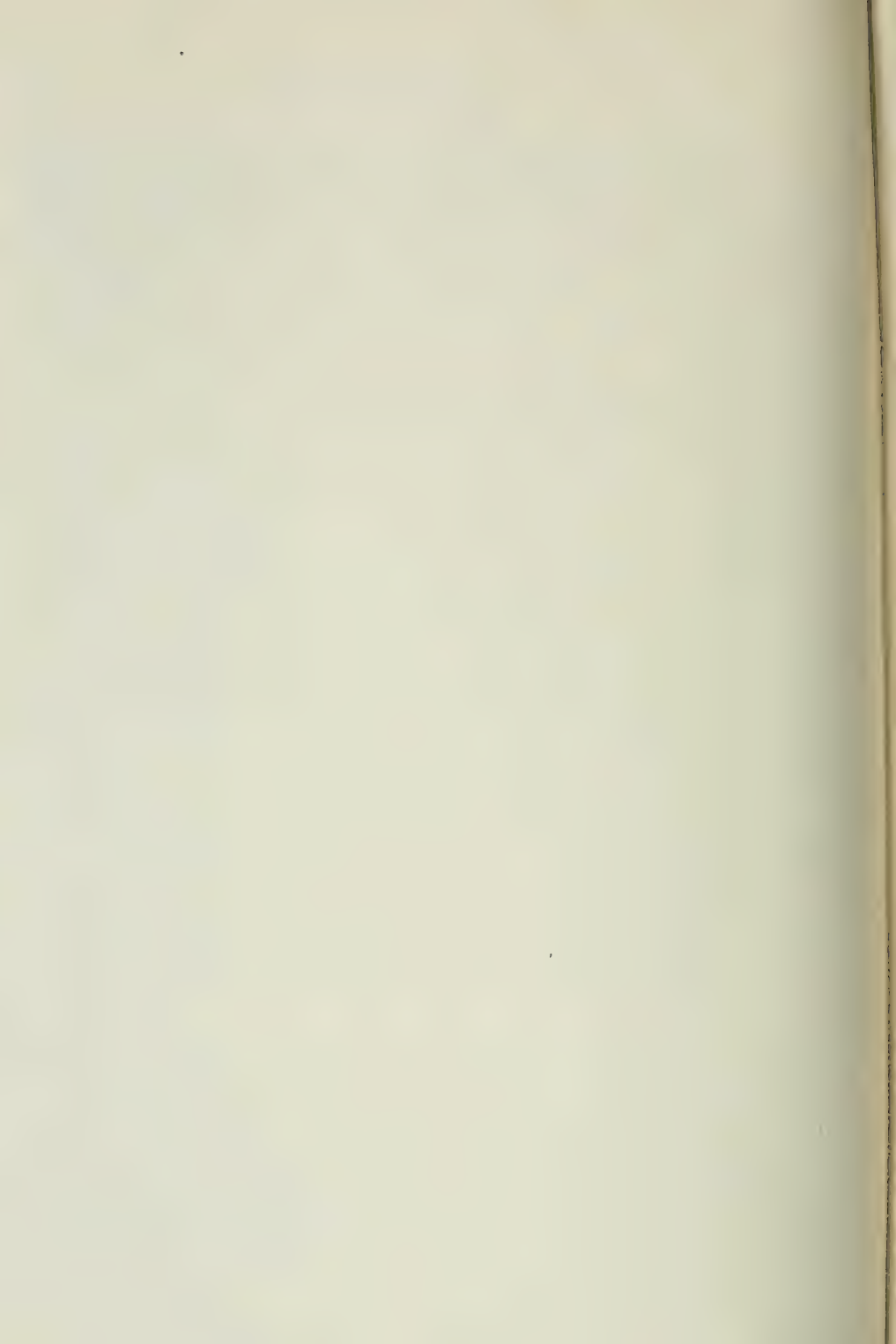
To recapitulate briefly: Four cases of pneumonia due to pneumococcus Type II were treated by oral insufflation of oxygen. In all cases the treatment began when the condition of the patients was already very grave and apparently hopeless. In all cases the cyanosis improved soon after the insufflation was started. In one case the cyanosis returned immediately after the insufflation was stopped. There was apparently no more storing capacity whatsoever for oxygen. That patient died one hour after the beginning of the treatment. In the second case there was at the beginning a definite storing capacity for oxygen which, however, gradually disappeared. The life

of that patient was then kept up for several hours by continually administering the (rhythmic) insufflation of oxygen. In a third case the storing capacity was small but remained unchanged for five hours. The patient died during a prolonged period when oxygen could not be readily procured. The fourth case, which practically was as hopeless as any of the foregoing, showed a definite reversion in the direction of recovery about two hours after the treatment by means of oral insufflation of oxygen was begun.

It seems justifiable to assume that the period of definite cyanosis is preceded by a more or less long period during which the capacities for taking up oxygen, and storing it, by the vital cells, are gradually getting impaired; and it is further justifiable to assume that during early pathologic periods the insufflation of oxygen may be capable of restoring these capacities to their normal extent more frequently and efficiently than during later stages. The early insufflation might thus be the means of preventing the disease from reaching the stage of cyanosis. In other words, early administration of oral insufflation in pneumonia and similar diseases may prove to be a real therapeutic measure. In normal conditions the individual inhales air which contains about 30 per cent oxygen above the physiologic need. This surplus presents the factor of safety for the healthy, moderately active individual. During sickness that gradually leads to respiratory insufficiency, the loss of the capacity of the vital body cells for taking up and storing oxygen, the factors of safety for this vital gas ought to be then considerably larger than in normal conditions. It is true that, experimentally, we shall be the losers. If the patient recovers under early administration of oxygen insufflation, we shall in most cases be deprived of the absolute evidence that the recovery was actually due to the treatment. But we must give the benefit of doubt to the patient.

I recommend, therefore, that in pulmonary and cardiac disease oxygen should be administered by means of the oral insufflation several times a day at an early period when there is not yet an urgent necessity for it. Oxygen should not be considered as a terminal measure. On the other hand, it ought to be emphasized that oxygen should not be considered as a specific, and its use should by no means eliminate the use of other promising remedies. On the contrary, oxy-

gen may make the body cells more amenable to the curative action of other therapeutic agents. Furthermore, the oral insufflations offer a chance to other therapeutic agents to develop their favorable action.



THE STRUCTURE OF YEAST NUCLEIC ACID.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 26, 1917.)

Levene and Jacobs have formulated the structure of yeast nucleic acid as a tetranucleotide. The facts that led up to their formulation were:

1. The formation of four nucleosides on neutral or ammonia hydrolysis.
2. The formation of simpler nucleotides on hydrolysis with dilute mineral acids.
3. The presence of a phosphorus to nitrogen ratio which agreed quite well for the tetranucleotide theory.
4. The ratio of amino to total nitrogen in nucleic acid was in harmony with the ratio required by the four bases, guanine, adenine, uracil, and cytosine.

The mode of linkage between the individual nucleotides was at that time not determined, and in the graphic formula representing the nucleic acid the linkage of the nucleotides was of a provisional character and arbitrary in nature.

Following that, the studies of Levene and Jacobs on thymus nucleic acid led to methods which permitted the separation and the study of individual mono- and dinucleotides composing the complex nucleic acids. Levene and Jacobs then returned to the study of yeast nucleic acid, applying the experience gained on the thymus nucleic acids. They then directed their attention to the nucleotides obtained by acid hydrolysis of the yeast nucleic acid. A large quantity of the material was prepared and transformed into the brucine salt. Other work, however, made a demand on their energies, and the work on the nucleotides was somewhat neglected. However, in course of the present academic year the work has been resumed. There were on hand 125.0 gm. of the brucine salts when the work was begun.

Meanwhile Dr. Walter Jones and his coworkers published several important publications on the structure of yeast nucleic acid. The basis of their work is the conception of the nucleic acid molecule as expressed by Levene and Jacobs.

The work of Jones deals specifically with the mode of linkage between individual nucleotides; Jones accepts a tetraribose of the structure $((C_5H_{10}O_5)_4 - 3H_2O)$ as the nucleus of the molecule. In this nucleus all the carbonyl groups of course must be free, since this is demanded by the existence of nucleosides. Dr. Jones bases his conclusion on two arguments: one is a proof by analogy; namely, the wide distribution of polysaccharides in nature; the other is the discovery by him of dinucleotides in which four of the hydroxyls of the phosphoric acid are free. The first argument does not seem valid, since according to present knowledge all the polysaccharides in nature have a glucosidic structure, and the one assumed by Jones to be present in the molecule of nucleic acid can be constructed only through ether linkage. Just because a proof by analogy is lacking, all the greater rigor is required from the experimental evidence.

There have appeared three papers by Jones and his coworkers dealing with the subject. In one, Jones and Richards claim to have cleaved the molecule of yeast nucleic acid into two large fractions, guanine-cytosine dinucleotide, and adenine-uracil dinucleotide. However, the authors admit that the dinucleotides were not isolated in pure form.

In a second paper Jones and Germann claim to have accomplished by ammonia hydrolysis the same cleavage as Jones and Richards had accomplished by enzymes. However, from the fraction named guanosine-cytosine only guanylic acid was obtained. The adenine-cytosine fraction analyzed for the dinucleotide and a brucine salt obtained from it analyzed satisfactorily for the same substance. The uracil nucleotide was not traced.

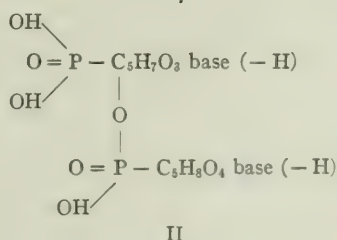
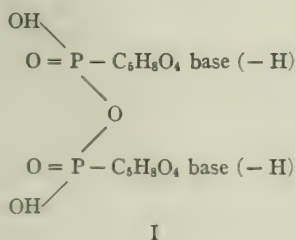
In a third paper Jones and Read, following closely the directions of Levene and Jacobs, prepared the pyrimidine nucleotide fraction previously obtained by these authors. Jones and Read converted it into the brucine salt and found the analytical data of this substance to agree with that of cytosine-uracil dinucleotide.

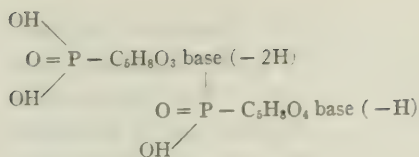
Thus the principal argument of Jones and his coworkers in favor

of the dinucleotide structure of their substances is based on the analysis of the brucine salts. The alkaloid brucine was introduced into the study of nucleotides by Levene and Jacobs. The advantage of this reagent, as seen by them, consisted in the fact that it permitted fractionation of the brucine salts of nucleotides not only out of water, but also out of ethyl or methyl alcohol. The separation of the cytosine and thymine nucleotides of the thymus nucleic acid was based on this property of the brucine salts. Not much importance was attributed to the analytical data of the brucine salts. Indeed, Jones did not exaggerate the value of the analytical data of the brucine salts when he criticized the work of Tannhauser. Employing the same ammonia hydrolysis Tannhauser thought he had isolated a trinucleotide. Jones took exception to the conclusion on the basis of the fact that the percentage composition of the brucine salt was not much different from that of the brucine salt of guanylic acid.

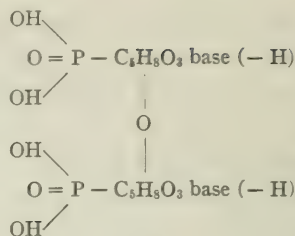
Levene and Jacobs, in their previous work on the brucine salts of nucleotides, formulated the following requirement as a test of the individuality of a nucleotide. First, a constant composition of the brucine salt on fractional crystallization, and second, a conversion of the brucine salt into a barium salt, which furnished analytical data agreeing with the theory for the assumed substance. This seemed the minimum requirement.

But even admitting that all the dinucleotides of Jones and coworkers actually exist, does this fact necessarily force the conclusion of a tetraribose nucleus? There are theoretically possible not only two but six ways of linkage between two nucleotides. They are:

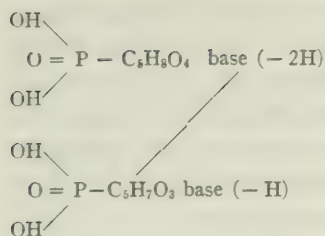




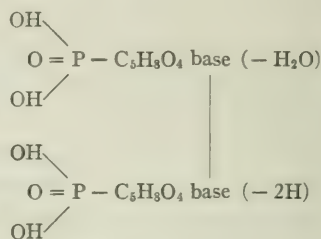
III



IV



V



VI

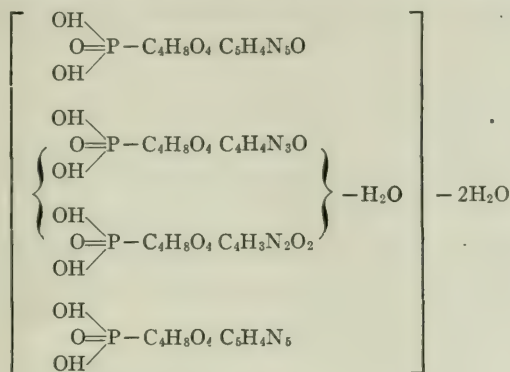
Forms III and V are possible of existence only when at least one of the two bases has two NH groups in the molecule and form VI when one base has two NH groups and the other, one NH and one OH groups.

In the first three forms of dinucleotides there are less than four readily ionizable hydrogen atoms. They should form di- or tribasic salts. But we have seen in guanylic acid the readiness with which a nucleotide forms a basic salt. Furthermore, dinucleotides of type II were shown to be present in thymus nucleic acid.

The remaining three may all function as tetrabasic acids. Forms IV and V may be regarded as the two most probable structures for dinucleotides forming tetrabasic salts. A decision between these two alternatives must be based on either experimental data or on valid theoretical reasoning.

These possibilities must be particularly borne in mind, because of the two following facts: first, in purine nucleotides the phosphoric acid is less firmly linked to the nucleoside than in pyrimidine nucleotides; and second, pyrimidine nucleotides form a dinucleotide more resistant toward hydrolytic action of acids than the purine nucleotides. The latter fact was first shown by Levene and Jacobs for

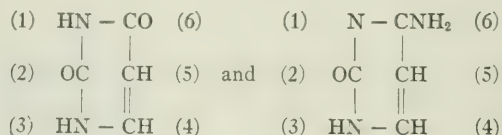
thymus nucleic acid, and is now shown to hold also for yeast nucleic acid. These differences in behavior are surely due to differences in structure. If a diribose is proven to exist in all dinucleotides, then the difference will have to be explained by the differences in the position of the hydroxyl groups which form the oxidic linking of two molecules of ribose. The final decision on the details of the structure of dinucleotides has to be reserved. All these considerations make it a difficult task to express the structure of yeast nucleic acid in a graphic formula without any arbitrary elements. If such a one is desired, it can only be expressed for the present in the following manner.



In the present communication it is desired to report on the cytosine-uracil dinucleotide prepared some time ago. The crude material was prepared nearly 5 years ago and the dinucleotide was isolated about 4 months ago. The result was not communicated because more light on the mode of linkage was desired. However, because of the recent publication of Jones and Read, our results are now presented. By means of fractional extraction with methyl alcohol the crude brucine salt was separated in several fractions, each different in composition. The fractions that came closest in their composition to that of the brucine salt of the cytosine-uracil dinucleotide were converted into a crystalline barium salt. The analytical data of the substance agreed with the theory for the assumed dinucleotide. The ratio of the amino to total nitrogen of the salt agreed with the theory for the barium salt of the dinucleotide. The melting point of our brucine salt was 200°C. (corrected). The physical constants of our

substance are slightly different from those of Jones and Read. This is not surprising since Levene and Jacobs have already shown that the crude barium salt is altered in composition by reprecipitation, and it is now also shown that various fractions of the crude brucine salt vary in composition. Furthermore, we are not aware of a single instance in which a partial hydrolysis leads to products of only one phase of hydrolysis. As a rule, under such conditions, substances of different degrees of cleavage are formed. Indeed, in the partial hydrolysis of thymus nucleic acid Levene and Jacobs have shown the presence simultaneously of thymine-cytosine dinucleotides and of the mononucleotides of the same bases. Whether the substance of Jones and Read or ours is nearest to the pure dinucleotide remains to be established.

As regards the mode of linkage of this dinucleotide, structure IV may perhaps be accepted, however, not with absolute certainty, as structure V cannot be excluded without further proof. However, if structure V were to be accepted it would necessitate the acceptance of a union between a hydroxyl group of the sugar and one of the nitrogen atoms of the base. Taking into consideration the structure of the two bases uracil and cytosine,



and further, taking into consideration the fact that in cytosine the NH_2 group is unsubstituted and that therefore cytosine cannot serve as a connecting link between two nucleotides, then uracil would have to be accepted as the bridge between two riboses of the dinucleotide. Future work will have to decide between the two structures. This applies also to the pyrimidine dinucleotide from thymus nucleic acid.

EXPERIMENTAL.

The bulk of the material was the brucine salts prepared about 5 years ago from the barium salts described by Levene and Jacobs.

50.0 gm. of the brucine salt were extracted with 1,500 cc. of boiling

methyl alcohol. The insoluble part was successively extracted until the insoluble residue did not perceptibly lose in weight on two successive extractions. The alcoholic extracts on standing at 25°C. formed a crystalline deposit. This was filtered, and the filtrate concentrated to dryness. The residue was recrystallized out of water. Out of 50.0 gm. there remained 14 gm. as the difficultly soluble part. This fractionation was carried out on two lots of 50.0 gm. each of the old material, and on two smaller samples prepared in course of this year. In the following table is given a summary of the analytical data of the various samples.

Sample No.	C	H	N	P	M. P.	$[\alpha]_D^{25}$
Insoluble.						
1.....	56.56	5.89	6.55	2.85	{ Contracted 178°C. Decomposed 215°C.	$\frac{-0.02 \times 10.0}{1 \times 0.05} = 4.0^\circ$
2.....	56.20	6.09	6.62	2.64		
3.....	56.72	6.18	6.11	3.17		
Soluble.						
4.....	58.18	6.37	8.05	2.70	{ Contracted 185°C. Decomposed 200°C.	$\frac{-0.05 \times 10.0}{1 \times 0.05} = -10.0^\circ$
5.....	58.99	6.68	7.89	2.79		
6.....	58.47	6.93	9.05	2.71	{ Contracted 185°C. Decomposed 200°C.	$\frac{-0.13 \times 5.0}{1 \times 0.05} = -13.0^\circ$
Theory for dinucleotide.....	59.36	6.73	8.19	2.80		

Samples 5 and 6 were dissolved in warm water with the aid of a slight excess of ammonia and shaken in a separatory funnel with chloroform to remove all brucine. To the brucine-free solution barium hydroxide was added in slight excess over the quantity theoretically required to neutralize the acidity of the nucleotide, and the solution was repeatedly evaporated to dryness under diminished pressure until all ammonia was removed. The residue was then dissolved in water and neutralized to litmus with sulfuric acid, filtered from barium

sulfate, and concentrated under diminished pressure to a very small volume until an insoluble white precipitate began to form. On cooling, the precipitate increased. It was filtered, dissolved to a clear solution, and again concentrated to a small volume. This time a granular precipitate settled out which under the microscope consisted of striated plates and needles.

0.0955 gm. substance required for neutralization 4.93 cc. 0.1 N acid.

0.1909 " " gave 0.0426 gm. $Mg_2P_2O_7$.

0.0955 " " " 0.0464 " $BaSO_4$

0.0966 " " " 0.0798 gm. CO_2 and 0.276 gm. H_2O .

0.0020 " " " in Van Slyke micro-apparatus 0.59 cc. N at $14^\circ C$. and 748 mm.

	Calculated for $C_{18}H_{21}N_5O_{16}P_2Ba_2+2H_2O$:	Found:
C.....	22.69	22.53
H.....	2.62	3.19
N.....	7.63	7.23
P.....	6.76	6.24
Ba.....	28.72	28.59
NH_2N	1.53	1.70

The optical rotation of the substance in 2.5 per cent HCl was:

$$[\alpha]_D^{25} = \frac{+0.50^\circ \times 2.5}{1 \times 0.100} = +12.5^\circ \text{ for the barium salt} = +18.52^\circ \text{ for the free dinucleotide.}$$

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THE REMOVAL OF NITRIC ACID FROM SOLUTIONS OF ORGANIC COMPOUNDS.

By P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 26, 1917.)

The lack of a convenient method for removing nitric acid has restricted to a minimum its use in organic and biological chemistry. In this laboratory we have felt the disturbing presence of nitric acid particularly in the preparation of the salts of anhydrotalonic and anhydrogalactonic acids, and of similar substances.

In inorganic analysis several methods for removing nitric acid are used. The majority of them are based on the process of reduction. Unfortunately the reduction is frequently carried out under conditions that would act destructively on the organic material, if any were present. The problem as it presented itself to us consisted in the selection of a method of reduction which could be carried out in a solution with a reaction in the neighborhood of neutrality and at ordinary temperature. Besides it was desired to select such reagents as were readily removable from the solution.

Of the reducing agents generally recommended for this purpose the following were employed: zinc dust, zinc-copper couple,¹ iron and sulfuric acid,² and aluminum amalgam.³ By means of zinc dust or zinc-copper couple in solutions approaching neutrality the reduction proceeded very slowly and never reached completion. On the other hand, by means of aluminum amalgam the reduction is completed in 6 hours. When the process of reduction of nitric acid by means of aluminum amalgam was recommended as a method for the quantitative determination of nitric acid, it was assumed that the acid was reduced completely to ammonia. Under the conditions here described only about 40 per cent of the nitric acid was reduced

¹ Williams, M. W., *J. Chem. Soc.*, 1881, xxxix, 100, 144.

² Alberti and Hempel, *Z. angew. Chem.*, 1892, 101.

³ Ormandy, R., and Cohen, J. B., *J. Chem. Soc.*, 1890, lvii, 811.

to ammonia; the remaining 60 per cent escaped the solution undoubtedly in the form of lower oxides of nitrogen. In this connection it was interesting to note that the rate of disappearance of nitric acid was much higher than that of ammonia formation, thus showing that in the course of the reduction the proportion of the lower oxides compared with that of N_2O_5 was continually increasing. Because of this it was deemed important to show experimentally that when the solution became free from nitric acid it was also free from N_2O_3 .

Theoretically one could not expect to find an absence of nitric acid where nitrous acid was present, since in aqueous solution, nitrites always assume the following equilibrium:



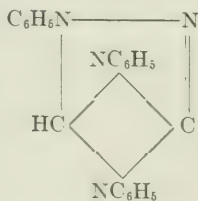
However, to remove all possible doubt, the reduced solution was analyzed for both nitric and nitrous acids.

Nitric acid was estimated by means of "nitron."⁴ At the end of the experiment a test for nitric acid was also made by means of brucine. The test was always negative.

The absence of nitrous acid was shown by means of a solution of potassium permanganate. After standing with the reduced solution for 1 hour no reduction of the permanganate solution could be detected. Hence it was proven that aluminum amalgam could be employed conveniently for removing nitric and nitrous acids.

When the process is applied to the removal of nitric acid from organic mixtures it becomes necessary also to remove the introduced

⁴ *Diphenyl-endanilo-dehydrotriazol*, with which it forms an insoluble precipitate.



reagents or their transformation product. The following procedure was adopted.

1. The acidity of the solution is determined by titration of a small sample and the solution is then neutralized by means of barium hydroxide.

2. About 2 gm. of freshly prepared aluminum amalgam are added for each gm. of nitric acid. The reduction is allowed to proceed for 8 hours or over night. The solution is aerated during the entire time of reduction.

3. The mixture is filtered from the mercury and aluminum. A slight excess of barium hydroxide is then added to the filtrate, and the mixture is concentrated under diminished pressure, to remove ammonia. Generally the process is completed after the evaporation has been repeated twice.

4. The barium is removed quantitatively and the filtrate is ready for further operations.

EXPERIMENTAL.

Determination of Nitric Acid with Nitron.—The solution of barium nitrate containing the equivalent of about 100 mg. of nitric acid was acidified with sulfuric acid and filtered from barium sulfate. To the filtrate 12 to 15 cc. of 10 per cent nitron solution in 5 per cent acetic acid were added and then the mixture was cooled in an ice bath. The precipitate was transferred to a weighed alundum crucible, washed with ice cold water, dried at 105–110°, and weighed. The weight of nitron nitrate $\times 0.168$ = nitric acid.

Determination of Ammonia.—Ammonia was determined by distilling aliquot portions of the solution, made alkaline, into 0.1 N acid. In those experiments in which the nitrate solutions were aerated during the reduction, the diluted acid solution into which the air was passed was added to the sample taken from the reaction flask, so that the determination in all experiments represents the total quantity of ammonia which was formed during the period indicated.

Aluminum Amalgam.—Sheets of aluminum foil about 4 by 6 inches are passed through a flame to remove the grease and immersed in a shallow bath of about 3 per cent solution of mercuric chloride. In a few minutes the surface of the foil is covered with mercury. The

foil is immediately washed in running water and is at once transferred to the nitrate solution.

Reduction of Nitrate Solutions.—Dilute solutions of barium nitrate were placed in flasks with aluminum amalgam. These flasks were fitted with rubber stopper and tubing so that the ammonia which might be generated would be collected in wash bottles containing dilute acid. In some experiments the ammonia was drawn into the dilute acid by a slow current of air.

Experiment 1.—4 gm. of barium nitrate were dissolved in 200 cc. of water and 2 gm. of aluminum amalgam were added and allowed to remain for about 2 hours without aeration. After 12, 36, and 60 hours, samples were withdrawn for analysis. The results are shown in Table I.

TABLE I.

Time.	Theory NH_3 obtainable	NH_3 found.	Theory.
<i>hrs.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
0	0.520		
12		0.0774	14.9
36		0.1449	27.9
60		0.0483	37.6

Experiment 2.—8 gm. of barium nitrate were dissolved in 400 cc. of water and 4 gm. of aluminum amalgam were added. This solution was aerated and the ammonia was determined after 12, 36, and 60 hours. The results are given in Table II.

TABLE II.

Time.	Theory NH_3 obtainable.	NH_3 found.	Theory.
<i>hrs.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
0	1.0400		
12		0.2734	27.0
36		0.1049	31.63
60		0.0219	38.00

Experiment 3.—2 gm. of barium nitrate were dissolved in 100 cc. of water, 1 gm. of aluminum amalgam was added, and aerated for 14

hours, after which time the nitric acid test with nitron was negative as well as with brucine.

Experiment 4.—8 gm. of barium nitrate were dissolved in 400 cc. of water and 6 gm. of aluminum amalgam were added. After 4 hours the nitric acid test with the nitron and brucine was negative.

Experiment 5.—50 cc. of 2.3 per cent nitric acid and 1 gm. of aluminum amalgam were allowed to stand for 16 hours. After 4 hours, 15.9 per cent acid was not reduced and after 16 hours 4.6 per cent nitric acid remained.

Experiment 6.—10 gm. of barium nitrate were dissolved in 500 cc. of water and 7 gm. of aluminum amalgam were added. Then additional amalgam was added every 2 hours as indicated in the table, and at each 2 hour interval a sample of the liquid was withdrawn for the determination of nitric acid and ammonia. As Table III shows, no nitric acid remained after 6 hours although only 40 per cent of the nitric acid was obtained in the form of ammonia.

TABLE III.

Nitric acid determined with nitrogen.						Ammonia (NH ₃).				
Time.	Aluminum amalgam.	Present.	Reduced.	Present.	Reduced.	Calculated from HNO ₃ present.	NH ₃ determined.	Total produced.	NH ₃ obtained during each interval.	NH ₃ obtained from beginning.
hrs.	gm.	gm.	gm.	per cent	per cent	gm.	gm.	gm.	per cent	per cent
0	7.0	5.660	0.000	100.0	000.0	1.527				
2	3.0	1.392	4.268	24.0	76.0		0.1205	0.1205	7.90	7.60
3	2.0	0.558	5.102	9.8	90.2		0.1475	0.2680	9.68	17.60
4	0.5	0.0045	5.665	0.79	99.21		0.1665	0.4345	10.90	28.50
5	0.5	Trace.	Nearly all.	Trace.	99.99		0.1904	0.6249	12.48	40.96
6	0.5	None.	5.660	None.	100.00					

Experiment 7.—10 gm. of barium nitrate were dissolved in 500 cc. of water and 7 gm. of aluminum amalgam were added. The solution was aerated and allowed to stand for 14 hours. The mixture was filtered and the filtrate tested for nitric and nitrous acids. Nitric acid was found absent both with brucine and nitron. 20 cc. of 0.1 N potassium permanganate acidified with sulfuric acid were added to

20 cc. of the filtrate and the unused permanganate was titrated, according to the method of Volhard, with potassium iodide. No nitrous acid was found.

THE PREPARATION OF LYXOSE.

By E. P. CLARK.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Of the methods for preparation of lyxose the one introduced by Ruff and Ollendorff¹ is the most convenient. This consists in the oxidation of calcium *d*-galactonate by means of hydrogen peroxide, using ferric acetate as a catalyst. The details of the directions as given by Ruff and Ollendorff are not sufficient for the preparation of this pentose on a large scale. In the course of the past year over 3,000 gm. of the sugar were prepared in this laboratory, and the process has been gradually improved so that finally a yield of 195 gm. of pure crystalline lyxose was obtained from 1,500 gm. of calcium galactonate. The conditions as finally adopted are reported here in the hope that they may prove useful to other workers.

EXPERIMENTAL.

500 gm. of calcium galactonate were dissolved in 2 liters of boiling water, and 3 liters of 3 per cent hydrogen peroxide added. The solution was cooled to about 35° and 75 cc. of ferric acetate solution² were added, which soon caused a vigorous reaction. After the reaction was completed, which was indicated by the solution acquiring a deep purple color, it was allowed to cool. The solution was filtered and evaporated in vacuum to about 1,200 cc. To the concentrated solution 4 liters of 95 per cent alcohol were added with constant stirring. This precipitates a gummy mass which is hard to handle but if a current of air is blown through the suspension for a short time, all the gummy particles settle out, leaving a clear solution. This solution was filtered with suction. The gum remaining in the jar, which

¹ Ruff, O., and Ollendorff, G., *Ber. chem. Ges.*, 1900, xxxiii, 1798.

² National Formulary, 3rd edition, Baltimore, 1906, p. 219.

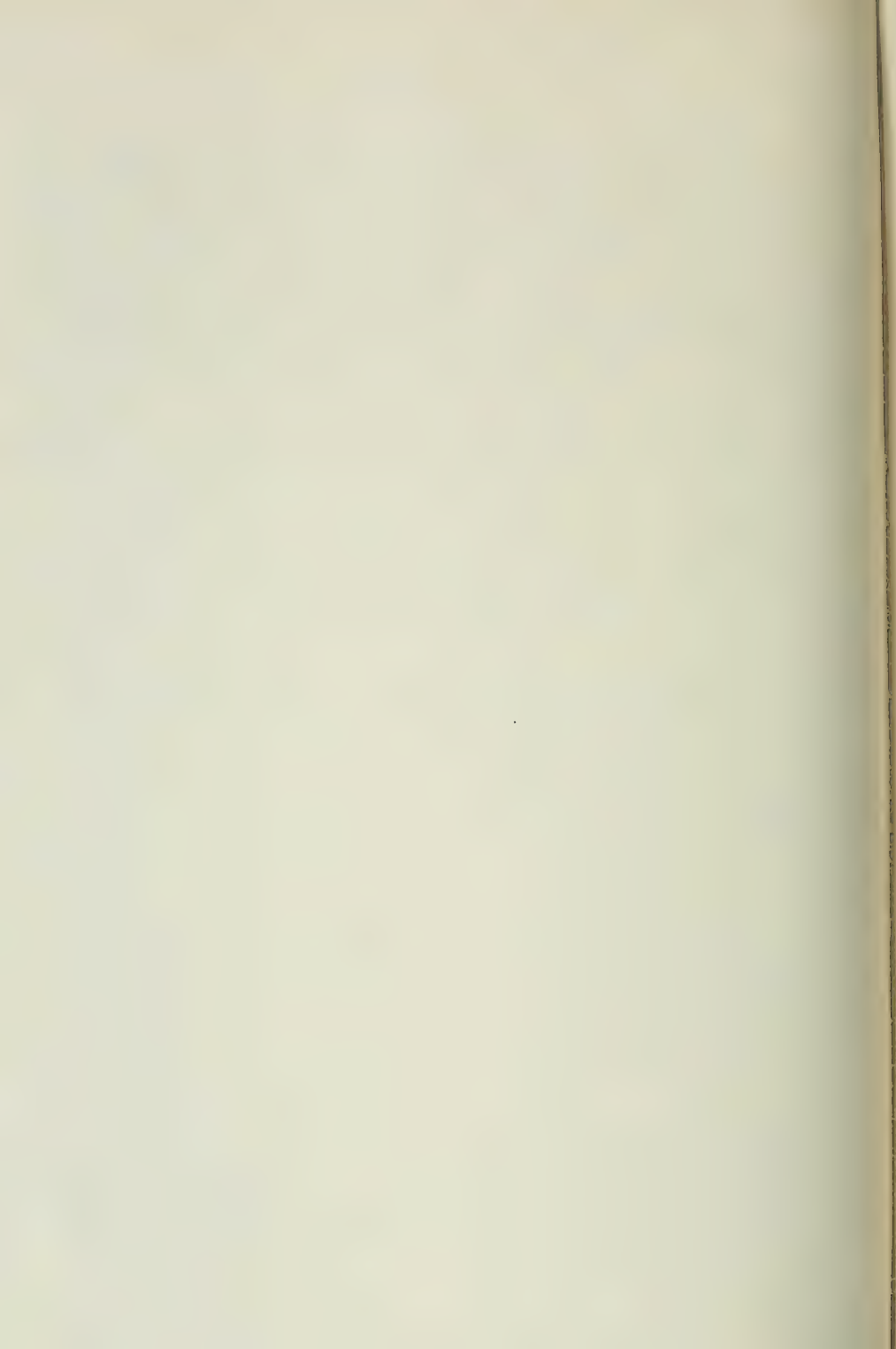
was drained as dry as possible, and the precipitate on the filter, were then dissolved in about 900 cc. of hot water. This may be readily accomplished in 4 or 5 minutes by heating the mixture, with constant stirring, to about 60–65° by means of live steam. After the gum had dissolved, the liquid was cooled to room temperature and precipitated with 4 liters of 95 per cent alcohol, as above.

Three lots of 500 gm. each were treated in this way and the combined residues from them were reoxidized in the following manner: They were dissolved in several liters of hot water, allowed to cool, and filtered. The filtrate was evaporated to dryness *in vacuo* to remove all the alcohol. The residue was then dissolved in about 2 liters of hot water by means of live steam and 5 liters of hydrogen peroxide were added. The solution was cooled to 35° and 80 cc. of ferric acetate solution were added. After the reaction was complete the solution was filtered and concentrated, then precipitated with 95 per cent alcohol as previously described. The residues were dissolved in 1 liter of water and again precipitated with 4 liters of 95 per cent alcohol.

The combined alcoholic extracts resulting from the above procedure were then evaporated *in vacuo* to 1 liter. 95 per cent alcohol was added with constant stirring until a permanent precipitate was formed; about 1.5 liters of alcohol were required. This solution was poured into 9 liters of absolute alcohol with constant agitation. The precipitate formed was filtered off, drained as dry as possible, and the filtrate evaporated *in vacuo* to a thick syrup (about 700 cc.). This syrup was taken up in 8 liters of absolute alcohol and 3.5 liters of dry ether *slowly* added with constant stirring, which precipitates a further quantity of calcium salts and other reaction products. The filtered solution was evaporated *in vacuo* to 500 cc., seeded with a few crystals of lyxose, and allowed to crystallize in a desiccator. Often the syrup can be made to crystallize spontaneously without seeding by scratching the inside of the beaker.

The crystals were filtered with suction and washed first with absolute alcohol and then with dry ether. The yield was generally 150 to 165 gm. of pure dry sugar. Lyxose may be readily recrystallized with little loss from four to five parts of boiling absolute alcohol. No attempt was made to work over the mother liquors from the

crystallization of lyxose, as it was found that they could be used directly for the preparation of lyxosimine. The syrup gave a yield of lyxosimine corresponding to about 55 or 60 gm. of lyxose. Yields of pure crystalline lyxose from lots of 1,500 gm. of calcium galactonate have been obtained as high as 195 gm., but in these cases a corresponding diminution of lyxosimine has been obtained from the mother liquors, so that almost invariably the total yield corresponded to about 210 gm. of lyxose from 1,500 gm. of calcium galactonate.



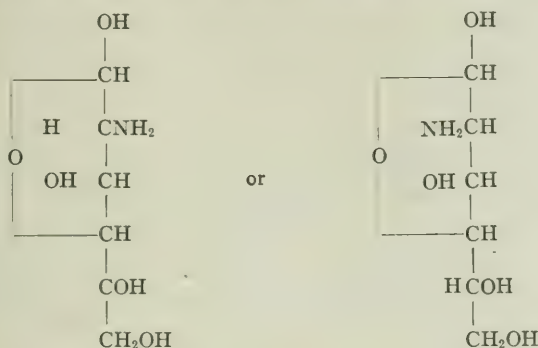
CHONDROSAMINE AND ITS SYNTHESIS.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 26, 1917.)

In previous publications¹ the conclusion was reached that chondrosamine had the structure of one of the two lyxohexosamines:



The relationship of chondrosamine to galactose was originally based on the following facts:

Chondrosamine formed with phenylhydrazine an osazone indistinguishable from galactosazone. Chondrosamine, on oxidation with bromine or with mercuric oxide, formed chondrosaminic acid. This acid on oxidation with nitric acid (subsequent to deamination) yielded anhydromucic acid. On the other hand direct oxidation of chondrosamine (subsequent to deamination) led to an optically active dicarboxylic acid which was assumed to be anhydrotalomucic acid. Furthermore, chondrosamine on oxidation with bromine or with mercuric oxide (subsequent to deamination) gave rise to anhydrotalonic acid. Chondrosaminic acid under the same treatment formed the isomeric anhydrogalactonic acid. In a measure the proof seemed sufficient to establish the configuration of the new aminohexose. Yet a scrutiny

¹ Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1913, xv, 159; 1914, xviii, 127, 240; 1915, xx, 434.

of the evidence makes it clear that only one point of evidence was absolutely beyond dispute; that is, the identity of the chondrosamine osazone with galactosazone. True, the two anhydrotetrahydroxyadipic acids, one obtained from chondrosamine and the other from lyxohexosaminic acids, seemed identical. However, this conclusion was based on their melting points being identical and on the absence of optical activity in one and the other. These points may be considered ample proof, but additional evidence was desirable. Again the anhydrotalonic and anhydrogalactonic acids had been prepared only in form of their brucine salts, and although the differences in their optical rotation were in full agreement with the assumed structure of the two salts, yet more direct evidence in support of the assumption was much wanted.

The most convincing proof possible would be of course the synthesis of all the substances derived from chondrosamine, using lyxose as the starting material. The synthesis of the sugar has now been accomplished, and with it for every known derivative of the natural sugar a corresponding derivative of the synthetic substance has been obtained. Thus the problem of the configuration of chondrosamine is definitely solved.

Chondrosamine Hydrochloride.—This was prepared last year by the reduction of lyxohexosaminic acid. Its optical rotation was then found to be $[\alpha]_D^{20} = +62.69$ to $+91.10^\circ$. The rotation of chondrosamine hydrochloride was determined by Levene and La Forge, $[\alpha]_D^{20} = +129.50$ to 93.82° . This difference in rotation and the fact that chondrosaminic acid differed in its rotation from the synthetic lyxohexosaminic acid led originally to the conclusion that the natural and the synthetic sugars were epimers. However, the fact that the rotation of the two sugars reached the same value at the state of equilibrium suggested the possibility that they were α and β forms of the same sugar. Hence the rotation of the recently prepared chondrosamine hydrochloride was redetermined, and was found identical with that of the synthetic product in the proximity of $[\alpha]_D^{20} = +57$ to 93° . Many samples crystalized under different conditions were tested, always with the same result. This was a surprising and puzzling finding; and although there was no doubt in our mind as to the correctness of the early measurement, since all readings in this lab-

oratory are taken by two observers, yet to eliminate all possible doubt the rotation of the original material was again measured and was found as originally recorded $[\alpha]_D^{20} = +129.0$ to 95.0° . It is interesting to note that in the early work of Levene and La Forge the first form occurred on three occasions whereas in subsequent work it could not be obtained again. It is also worthy of note that the value of the molecular rotation of the end carbon atom of the two amino sugars calculated according to Hudson's formula is in good agreement with the value calculated by Hudson for the end carbon atom of other hexoses.

Chondrosaminic Acid.—The observations on chondrosaminic acid and on the acid obtained synthetically originally seemed puzzling and confusing. By the action of hydrocyanic acid on arabinosimine, Fischer and Leuchs² obtained pure glucosaminic acid. Because of this it was thought that lyxohexosaminic acid obtained by the action of hydrocyanic acid on lyxosamine was also a uniform substance. The synthetic acid originally obtained in this manner had $[\alpha]_D^{20} = -3.58$ to -20.7° , which differed from chondrosaminic acid with $[\alpha]_D^{20} = -16.15$ to -29.2° . On the other hand, the reduction of the synthetic acid led to the same chondrosamine, which on oxidation gave rise to chondrosaminic acid. The confusion, however, was cleared up when it was found that the synthetic sugar on oxidation formed the same amino acid as the natural sugar.

Parallel measurements of the optical rotation were made on samples of the acids from the natural and synthetic sugars and were found identical, $[\alpha]_D^{20} = -17.94$ to -31.89° . In the light of this, lyxohexosaminic acid has to be regarded as a mixture of the epimeric acids. Indeed, the material prepared this year had $[\alpha]_D^{20} = -1.85$ to -8.78° , which differed from the $[\alpha]_D^{20}$ of the original synthetic acid. Obviously under varying conditions of experiment different proportions of the epimeric acids are formed.

α , α_1 -Anhydromucic Acid, and α , α_1 -Anhydrotalomucic Acids.—Inactive anhydrotetrahydroxyadipic acids were obtained from chondrosamine and from the synthetic lyxohexosaminic acids. So long as the latter was considered an individual substance different from chondro-

² Fischer, E., and Leuchs, H., *Ber. chem. Ges.*, 1903, xxxvi, 24.

saminic acid, the observation needed special interpretation. Since, however, it was demonstrated that the synthetic acid was a mixture of two epimers, one being chondrosaminic acid, the puzzling element of the observation was eliminated, the identity of the two anhydrotetrahydroxyadipic acids became evident, and their structure could be no other than that of anhydromucic acid.

The structure of the optically active tetrahydroxyadipic acids is manifested from the fact that the natural sugar gives rise to the acid with exactly the same properties as that derived from the synthetic sugar. Since the structure of the latter is obvious from its origin the structure of the former as anhydrotalomucic becomes certain.

α, α_1 -Anhydrotalonic and α, α_1 -Anhydrogalactonic Acids.—Since the structure of the α, α_1 -anhydrotalomucic and α, α_1 -anhydromucic acid has been established it became an easy task to prove the structure of the two monocarboxylic acids. The anhydrotalonic should be convertible into anhydrotalomucic acid, and anhydrogalactonic into anhydromucic acid. The brucinesalts of two acids were previously obtained, one with a melting point of 218° and $[\alpha]_D^{20} = -12.4$, and the other with a melting point of 244° and $[\alpha]_D^{20} = -9.40$. It was then shown that the former on oxidation gives anhydrotalomucic while the latter forms anhydromucic acid. The former was assumed to possess the structure of anhydrotalonic, the latter of anhydrogalactonic acid. The assumption was fully justified and stands correct in the light of the new evidence.

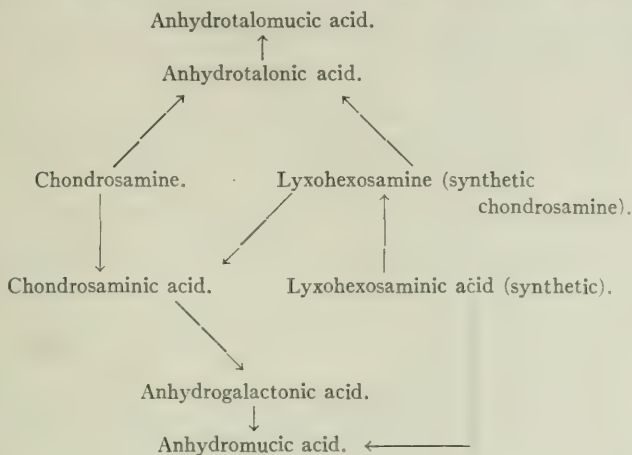
It may be mentioned in this place that formerly the formation of anhydrotalonic acid from the synthetic lyxohexosaminic had seemed puzzling. In view of the fact that lyxohexosaminic acid is recognized as a mixture of two epimers, the source of the anhydrotalonic is to be looked for in one of the two epimers, namely, in epichondrosaminic acid.

Pentacetyl Derivatives.

As a final point proving the identity of the two sugars may be mentioned the fact that they yield the identical pentacetates. Both the α and the β forms obtained by Hudson and Dale from the natural product were then obtained from the synthetic sugar. It may be noted in this place that Hudson and Dale designated the more soluble pent-

acetate of chondrosamine with $[\alpha]_D^{20} = 101.3^\circ$ as the β form and the more insoluble with $[\alpha]_D^{20} = 11.0^\circ$ as the α form. This nomenclature was perfectly justified when it was assumed that chondrosamine was *l*-ribohexosamine. Inasmuch as chondrosamine is now recognized as *d*-lyxohexosamine, the form with the higher rotation has to be named the α form, $[\alpha]_D^{20} = 101.3^\circ$, and the one with $[\alpha]_D^{20} = +11.0$, the β form.

The mutual relationship of the derivatives of chondrosamine on one hand and of the derivatives and of the parent substances of the synthetic sugar on the other is represented in the following diagram.



EXPERIMENTAL.

Preparation of Synthetic Chondrosamine Hydrochloride.—The process was essentially the same as previously described.³ A slight improvement was introduced in that all solutions were concentrated at a temperature of the water bath not exceeding 40°C . It was found convenient to recrystallize the sugar by dissolving it in a minimum amount of water and adding ethyl alcohol saturated with hydrochloric acid. About 35.0 gm. of the synthetic sugars were prepared. The specific rotation of the substance was the following.

Initial.	Equilibrium.
$[\alpha]_D^0 = \frac{+1.50 \times 2.0392}{1 \times 0.0516} = +59.30^\circ$	$[\alpha]_D^{25} = \frac{+2.50 \times 2.0392}{1 \times 0.0516} = +98.80^\circ$

³ Levene, P. A., *J. Biol. Chem.*, 1916, xxvi, 143, 155.

α and β Forms of Chondrosamine Hydrochloride.—A sample of chondrosamine recrystallized out of water and ethyl alcohol saturated with hydrochloric acid.

$$\begin{array}{ll}
 \text{Initial.} & \text{Equilibrium.} \\
 [\alpha]_D^{25} = \frac{+1.54 \times 2.0385}{1 \times 0.0516} = +53.14 & [\alpha]_D^{25} = \frac{+2.28 \times 2.0392}{1 \times 0.0514} = +90.42
 \end{array}$$

A sample was dissolved in a minimum amount of water, glacial acetic acid was added until the substance began to crystallize, the mixture brought to a boil, and filtered. A solution was made of exactly 2.5 cc. volume.

$$\begin{array}{ll}
 \text{Initial.} & \text{Equilibrium.} \\
 [\alpha]_D^{25} = \frac{+3.04 \times 2.5}{1 \times 0.125} = +60.4^\circ & [\alpha]_D^{25} = \frac{4.5 \times 2.5}{1 \times 0.125} = +90.0^\circ
 \end{array}$$

A sample crystallized out of a minimum amount of aqueous hydrochloric acid.

$$\begin{array}{ll}
 \text{Initial.} & \text{Equilibrium.} \\
 [\alpha]_D^{25} = \frac{+2.60 \times 2.5}{1 \times 0.1272} = +57.10^\circ & [\alpha]_D^{25} = \frac{+4.80 \times 2.5}{1 \times 0.1272} = +94.20^\circ
 \end{array}$$

No attempt was made to explain the slight discrepancies in the solutions since the principal object was to determine conditions controlling the formation of either one of the forms. As all attempts to obtain a sample with the original rotation failed, the rotation of the original material was redetermined. Dr. J. López-Suárez and Dr. G. M. Meyer controlled the reading.

$$\begin{array}{ll}
 \text{Initial.} & \text{Equilibrium.} \\
 [\alpha]_D^{25} = \frac{+2.50 \times 2.5}{1 \times 0.0500} = +129.0^\circ & [\alpha]_D^{25} = \frac{+1.90 \times 2.5}{1 \times 0.500} = +96.0^\circ
 \end{array}$$

Calculating on the basis of Hudson's formula, the molecular rotation of the end carbon atom = $\frac{(129.0 - 51.0)}{2} 215.5 = 8,400$. The value found by Hudson for hexoses was in the neighborhood of 8,000. The original form is to be regarded as the α and the new as the β form.

Chondrosaminic Acid from Natural and Synthetic Sugars.—This acid was obtained from both the natural and synthetic sugar by oxidation with mercuric oxide. The conditions given by Pringsheim and Ruschmann⁴ for oxidation of glucosamine had to be modified.

4.0 gm. of the synthetic sugar were dissolved in 62.0 cc. of water, to the solution 20.0 gm. of mercuric oxide were added, and the mixture was warmed on the water bath for 6 minutes. The reaction product was filtered immediately, the filtrate was freed from mercury by means of hydrogen sulfide, the filtrate from the sulfide concentrated to a small volume, under diminished pressure, when the acid crystallized in the distilling flask. The substance was recrystallized once. It did not melt, but turned light brown at 190°, the same as the acid obtained from the natural sugar. A parallel measurement of the rotation of each product gave the following results.

Natural product.

Initial in 2.5 per cent HCl.	Equilibrium.
$[\alpha]_D^{25} = \frac{-0.90 \times 2.5}{1 \times 0.1254} = -17.94^\circ$	$[\alpha]_D^{25} = \frac{-1.60 \times 2.5}{1 \times 0.1254} = -31.89^\circ$

Synthetic.

Initial in 2.5 per cent HCl.	Equilibrium.
$[\alpha]_D^{25} = \frac{-0.94 \times 2.5}{1 \times 0.1256} = -17.94^\circ$	$[\alpha]_D^{25} = \frac{-1.60 \times 2.5}{1 \times 0.1256} = -31.87^\circ$

Lyxohexosaminic acid.

$[\alpha]_D^{25} = \frac{+0.37 \times 15.0}{2.0 \times 1.496} = +1.85^\circ$	$[\alpha]_D^{25} = \frac{-1.73 \times 15.0}{2.0 \times 1.4946} = -8.75^\circ$
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The analysis of the synthetic acid gave the following results.

0.1050 gm. of substance gave 0.1426 gm. CO₂ and 0.0618 gm. H₂O.

	Calculated for C ₆ H ₁₃ NO ₈ :	Found:
C.....	36.92	37.03
H.....	6.66	6.58

Thus the identity of the two chondrosaminic acids is established.

Anhydrotalonic Acid.—The acid was previously obtained in small quantities as a brucine salt. The brucine salt was prepared in larger quantities from the natural sugar and from the sugar obtained from

⁴ Pringsheim, H., and Ruschmann, G., *Ber. chem. Ges.*, 1915, xlviii, 680.

lyxohexosaminic acid. Lots of 30.0 gm. of chondrosamine hydrochloride were dissolved in 150.0 cc. of water. To the solution 30.0 gm. of silver nitrite and a few drops of hydrochloric acid were added. The mixture was allowed to react for 6 hours. The silver chloride was then removed by filtration, the filtrate was placed on a boiling water bath for 5 minutes, then treated with a slight excess of hydrochloric acid, and again filtered. To the filtrate 65.0 gm of bromine were added and the mixture was allowed to stand at room temperature for 3 days. The remaining traces of bromine were removed by shaking the solution with mercury, and the hydrobromic acid by means of lead carbonate and silver carbonate. Finally the nitric acid still present in the solution was removed by the aluminum amalgam method. The resulting solution was transformed into the brucine salt in the usual manner. The solution of the brucine salt was concentrated to a small volume when, on cooling, the brucine salt was crystallized out. When first obtained the substance was readily soluble in methyl alcohol. However, on repeated recrystallization its solubility diminished, so that towards the end it dissolved only in a large volume of boiling methyl alcohol. The salt then crystallized in large polygonal prisms. The melting point was 218°C. and the optical rotation was the following.

$$[\alpha]_D^{20} = \frac{-0.63^\circ \times 10.0}{2 \times 2.522} = -12.4^\circ$$

The analysis of the substance gave the following results.

0.1063 gm. of substance on drying under diminished pressure at the temperature of xylene vapors lost 0.003 gm. of water.

	Calculated for $C_{22}H_{36}N_2O_{10} + H_2O$:	Found:
H ₂ O.....	3.11	3.58

0.1025 gm. of the dry substance gave 0.2284 CO₂ and 0.0569 gm. H₂O.

	Calculated for $C_{29}H_{36}N_2O_{10}$:	Found:
C.....	60.80	60.78
H.....	6.10	6.15

From the synthetic lyxohexosaminic acid the substance was prepared in the following manner. 30 gm. of the acid were dissolved in a solution of 200.00 cc. of water and 40.0 cc. of 10 per cent hydrochloric acid. 40.0 gm. of silver nitrite were added. The following

morning 10.0 gm. of silver nitrite and 10.0 cc. of 10 per cent hydrochloric acid were added. After 30 hours from the beginning of the experiment, the silver chloride was removed by filtration and other remaining silver by hydrogen sulfide. From the filtrate the nitric and nitrous acids were removed by the aluminum amalgam method.

It had the following composition.

0.1012 gm. of substance on drying in a xylene bath under diminished pressure lost 0.0038 gm. of H_2O .

0.0974 gm. of substance, dried, on combustion gave 0.2158 gm. CO_2 and 0.0562 gm. H_2O .

	Calculated for $\text{C}_{29}\text{H}_{46}\text{N}_7\text{O}_{10}\text{H}_2\text{O}$:	Found:
H_2O	3.11	3.75
	Calculated for $\text{C}_{29}\text{H}_{46}\text{N}_7\text{O}_{10}$:	Found:
C.....	60.80	60.42
H.....	6.10	6.45

The substance had a melting point of 218°C . and the following rotation.

$$[\alpha]_D^{20} = \frac{-0.655^\circ \times 10}{2 \times 2.66} = -12.3$$

Thus the identity of the acid obtained from natural chondrosamine and of the one obtained synthetically was established. In order to prove its identity with α , δ -anhydrotalonic acid it was oxidized by means of nitric acid. 21.0 gm. of the brucine salt were freed from brucine by means of barium hydroxide and chloroform. After the removal of barium, the solution was concentrated to 40.0 cc., an equal volume of concentrated nitric acid was added, and the solution was boiled over a free flame until a lively evolution of red fumes set in. The solution was then transferred to a large clock glass and evaporated to dryness. The residue was dissolved in water and again evaporated to dryness. This operation was repeated. The reaction product was converted into the calcium salt. The salt was recrystallized twice. Each time the calcium salt was decomposed by a little less than the calculated amount of oxalic acid, and then reconverted into the calcium salt.

0.1008 gm. of the air-dry substance on drying in a xylene bath under diminished pressure lost 0.0130 gm. of H_2O .

	Calculated for $\text{C}_6\text{H}_{10}\text{O}_8\text{C}_6 + 3\text{H}_2\text{O}$:	Found:
H_2O	12.68	12.90

0.0878 gm. of dry substance gave on combustion 0.0936 gm. CO_2 and 0.0274 gm. H_2O .

0.0896 gm. of the substance gave 0.0206 gm. CaO .

	Calculated for $\text{C}_6\text{H}_8\text{O}_7\text{Ca} + \text{H}_2\text{O}$:	Found:
C.....	29.03	29.07
H.....	3.22	3.49
Ca.....	22.58	22.99

The optical rotation of the substance in a 10 per cent solution of HCl was the following.

$$[\alpha]_{\text{D}}^{20} = \frac{-0.30^\circ \times 2.50}{1 \times 0.100} = -7.5^\circ$$

Thus the anhydrohexonic acid obtained from lyxohexosaminic acid yields on further oxidation an optically active anhydrotetrahydroxyadipic acid; hence it possesses the structure of anhydrotalonic acid.

Anhydrogalactonic Acid.—10 gm. lots of chondrosaminic acid were treated in the same manner as lyxohexosaminic acid in the above experiment. The brucine salt obtained in this manner melted at 244° , and had the following optical rotation.

$$[\alpha]_{\text{D}}^{20} = \frac{-0.47^\circ \times 10.0}{2 \times 2.508} = -9.37^\circ$$

The composition of the substance was the following.

0.0988 gm. of the substance, dried in a xylene bath, gave 0.2196 gm. CO_2 and 0.0576 gm. H_2O .

	Calculated for $\text{C}_{23}\text{H}_{25}\text{N}_5\text{O}_{10}$:	Found:
C.....	60.80	60.73
H.....	6.10	6.53

10.0 gm. of the brucine salt were freed from brucine as in the above experiment. The brucine-free solution was evaporated to 25 cc. and then diluted with an equal volume of concentrated nitric acid and boiled over free flame until the volume was reduced to about 20.0 cc., then 10.0 cc. of nitric acid were again added, and the solution was again boiled, over free flame. When the solution was concentrated

to 20.0 cc. it was transferred to a clock glass and concentrated on a boiling water bath to dryness. The substance immediately crystallized. It was redissolved in water and again evaporated. The operation was repeated once more. The final crystalline residue was dissolved in hot acetone and very little ether was added, when a small amorphous precipitate formed. This was removed by filtration, and the filtrate was allowed to crystallize. The crystals were filtered, redissolved in hot acetone, and allowed to crystallize. The final product melted at 205°C.

0.1600 gm. of the substance dissolved in 2.5 cc. showed no optical activity.

On the basis of this the identity with anhydrogalactonic acid of the monohydroxy acid obtained from chondrosaminic acid is established.

α, α_1 -Anhydrotalomucic Acid from the Synthetic Sugar.—6.0 gm. of the synthetic lyxohexosamine hydrochloride were dissolved in 30.0 cc. of water containing 1 cc. of hydrochloric acid. 8.0 gm. of silver nitrate were added and the mixture was allowed to stand for 6 hours. It was then filtered. The silver was removed from the filtrate by means of hydrogen sulfide. The clear filtrate from the silver sulfide was concentrated to 20.0 cc., cooled to 0°C., and diluted with 20.0 cc. of nitric acid also cooled at 0°C. The solution was allowed to stand over night. It was then boiled over a free flame until a lively evolution of yellow fumes set in. The solution was then transferred to a clock glass, evaporated on a water bath, and the calcium salt was then prepared in the usual way. The salt was reprecipitated twice and had the following composition.

0.1054 gm. of substance on drying in a xylene bath lost 0.0130 gm. H_2O .

	Calculated for $C_6H_8O_8Ca + 2H_2O$:	Found:
H_2O	12.68	12.33

0.0924 gm. of the dry substance gave on combustion 0.0982 gm. CO_2 and 0.0298 gm. H_2O and 0.0924 gm. CaO .

	Calculated for $C_6H_8O_7Ca + H_2O$:	Found:
C	29.03	28.98
H	3.22	3.60
Ca	22.58	23.05

The optical rotation of the substance is the following.

$$[\alpha]_D^{20} = \frac{\text{Initial.}}{1 \times 0.100} = -8.0^\circ \quad [\alpha]_D^{20} = \frac{\text{Equilibrium.}}{1 \times 0.100} = -8.0^\circ$$

in 10 per cent HCl.

Hence the substance is α, α_1 -anhydrotalomucic acid. It is identical with the substance previously obtained from natural chondrosamine.

Pentacetyl Derivative of the Synthetic Chondrosamine.—The substance was prepared from the natural chondrosamine by Hudson and Dale.⁵ Practically the same conditions were followed in this work.

In 30.0 cc. of acetic anhydride 5.0 gm. of zinc chloride were dissolved. To this solution 5.0 gm. of the hydrochloride were added and the mixture was warmed gently until a lively reaction developed. The reaction was kept up for 2 minutes, then the reaction product was poured into 100 cc. of water cooled to 0°C. The mixture was neutralized with potassium bicarbonate, transferred to a separatory funnel, and extracted with chloroform. The chloroform extract was washed with water. Over the chloroform a layer of crystals appeared which were insoluble in water. The crystals consisted of the more insoluble fraction of the pentacetates. The chloroform extract was evaporated to dryness *in vacuo*, the residue was recrystallized out of alcohol, and from the mother liquor a second crop of crystals was obtained. The top fraction consisted of the pure α form while the most soluble form was practically the pure β form.

The α form turned slightly brown at 232° and melted with decomposition at 237°C. (corrected). Its optical rotation in chloroform solution was the following.

$$[\alpha]_D^{20} = \frac{+0.07 \times 20.0}{2 \times 0.0802} = +8.75^\circ$$

A similar fraction from natural chondrosamine had a melting point of 235°C. and the following optical rotation.

$$[\alpha]_D^{20} = \frac{0.09 \times 20.0}{2 \times 0.075} = +12.0^\circ$$

Hudson and Dale found for their β form a melting point of 235° (with decomposition) $[\alpha]_D = +11.00^\circ$.

⁵ Hudson, C. S., and Dale, J. K., *J. Am. Chem. Soc.*, 1916, xxxviii, 1431

The β form was apparently not quite pure, but taking into consideration the small quantity of starting material it is rather surprising that each form could be separated with so little difficulty.

The melting point of the β form was very sharp at 197°C . and the optical rotation in chloroform solution was

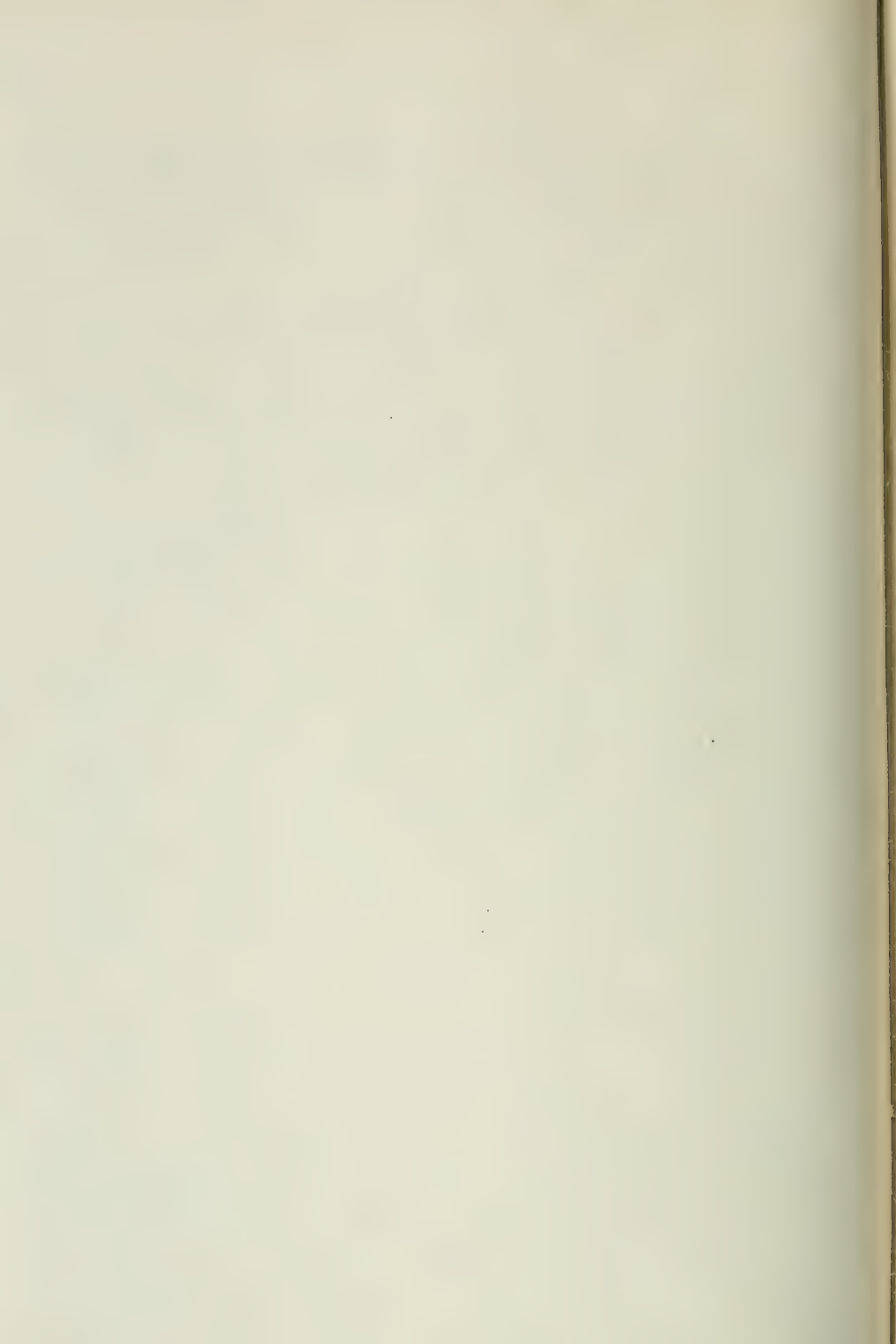
$$[\alpha]_D^{20} = \frac{+0.90 \times 20.0}{2 \times 1.000} = +90.0^{\circ}$$

Hudson and Dale found for the α form the melting point $182\text{--}183^{\circ}\text{C}$. and $[\alpha]_D$ 101.3° .

The composition of the pentacetyl derivative was the following.

0.1014 gm. of the substance gave 0.1834 gm. CO_2 and 0.0560 gm. H_2O .

	Calculated for $\text{C}_6\text{H}_8\text{NO}_5(\text{CH}_3\text{CO})_5$:	Found:
C.....	49.49	49.32
H.....	5.96	6.18



THE RELATION BETWEEN THE CONFIGURATION AND ROTATION OF EPIMERIC MONOCARBOXYLIC SUGAR ACIDS.

III. THE PHENYLHYDRAZIDES.

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In previous publications¹ it was demonstrated that the direction of the optical rotation of the α carbon atom of a pair of epimeric monocarboxylic sugar acids is determined by its configuration. When the hydroxyl of the α carbon atom is in the same position as in *d*-gluconic acid (on the right) the direction of the rotation is right, while when it is in the same position as in *d*-mannonic acid the direction of the rotation is left. The rule was found valid for phenylhydrazides, brucine, strychnine, and sodium and calcium salts.

In the early phase of the work it seemed that in salts of different acids with the same base and possibly in all derivatives of the sugar acids, the value of the molecular rotation of the α carbon atom was constant. If that were true the magnitude of the rotation of the α carbon atom of a given salt would serve as an index of its purity. Following our observation, Hudson² succeeded in demonstrating that in phenylhydrazides the magnitude of the rotation of the β , γ , and δ carbon atoms of several monocarboxylic acids were insignificant as compared with that of the α carbon atom, so that the direction of the rotation of the phenylhydrazide could be determined by that of the α carbon atom. Because of this Hudson suggested that, *vice versa*, the configuration of the α carbon atom in a given sugar acid may be determined by the direction of the rotation of its phenylhydrazide. Wherever the phenylhydrazide is obtainable this undoubt-

¹ Levene, P. A., *J. Biol. Chem.*, 1915, xxiii, 145. Levene, P. A., and Meyer, G. M., *ibid.*, 1916, xxvi, 355.

² Hudson, C. S., *J. Am. Chem. Soc.*, 1917, xxxix, 462.

edly is correct. In our first note on this subject only one pair of epimeric phenylhydrazides was discussed, and further work was in progress when Hudson's article appeared. We did not discontinue our work for the reason that it has furnished some interesting results regarding the numerical value of the α carbon atom in different derivatives of the same acid.

As already mentioned, it was expected that the molecular rotation of the α carbon atom would be constant in all compounds of such acids, the purity of which could not be questioned. A scrutiny of the empirical values for the molecular rotation of the α carbon atom for the various pairs of epimeric salts revealed considerable variations, and it remained uncertain whether these were occasioned by the impurity of some of the acids.

The maximum rotation of the α carbon atom of a pair of acids is obtained when both acids are perfectly free from the epimeric acid, since the rotation of the α carbon atom of each of two epimers is in opposite directions. There can scarcely be any doubt that derivatives of gluconic and mannonic acids can be obtained in perfect purity, since both acids form crystalline lactones. Hence it was surprising to find that the rotation of the α carbon atom in the pair of allonic and altronic hydrazides was of a higher value than that of the gluconic-mannonic pair. On the other hand, in the gulonic-idonic pair the magnitude of rotation was equal to that of the gluconic-mannonic pair. Hence it is justifiable to accept the purity of idonic acid. On the other hand, the value of the rotation of the α carbon atom in the talonic-galactonic pair was much lower than that of any other pair. Besides, the rotation of both hydrazides was found to be in the same direction. Since galactonic lactone is obtained in a beautifully crystalline form, there exists no doubt as to its purity. On the contrary, the hydrazide of talonic acid was obtained from the brucine salt, and there always existed among workers a certain scepticism as to the purity of this compound. The brucine salt employed in this work has the rotation of $[\alpha]_D^{20} = -26.15^\circ$. This is the highest value recorded for the rotation of the brucine salt of talonic acid.

The scrutiny of the data obtained in the course of this work indicates that the magnitude of the rotation of the α carbon atom is not altogether constant even for the series of phenylhydrazides, but the

rule of the relation of direction of the rotation to the configuration of the α carbon atom remains valid.

In the present work all readings were made on two solutions prepared from the same sample so as to reduce to a minimum the experimental error.

Phenylhydrazide.	Author.	C	T°	$[\alpha]$	$\frac{A+B}{2}$
Gluconic.	Nef. ³	1	85	+18.0	14.25°
		1	85	+18.2	
		2	20	+12.0	
		1	85	-10.5	
		1	90	-10.7	
		2	80	- 8.1	
Mannonic.	Hudson. ²	1	20	+13.45	14.25°
		1	20	+13.87	
		4	20	+13.74	
		1	20	-15.1	
		1	20	-15.3	
		2	20	-12.42	
Gulonic.	Nef.	1	25	+12.2	8.25°
		2	20	+10.44	
		0.5	25	+ 4.35	
		1	20	+25.88	
		1	20	+25.50	
		1	20	-15.8	
Idonic.	Nef.	1	20	-15.9	20.8°
		1	20	-15.9	
		1	20	-15.9	
		1	20	-15.9	
		1	20	-15.9	
		1	20	-15.9	
Galactonic.	Nef.	1	25	+12.2	8.25°
		2	20	+10.44	
		0.5	25	+ 4.35	
		1	20	+25.88	
		1	20	+25.50	
		1	20	-15.8	
Talonic.	Nef.	1	20	-15.9	20.8°
		1	20	-15.9	
		1	20	-15.9	
		1	20	-15.9	
		1	20	-15.9	
		1	20	-15.9	
Allonic.	Hudson.	2.5	63	-16.09	16.09°
		1	20	-14.5	
		2.5	63	+16.09	
		2.5	63	+16.09	
		2.5	63	+16.09	
		2.5	63	+16.09	
Altronic.	Hudson.	2.5	63	-16.09	16.09°
		1	20	-14.5	
		2.5	63	+16.09	
		2.5	63	+16.09	
		2.5	63	+16.09	
		2.5	63	+16.09	
Arabonic.	Hudson.	2.5	63	-16.09	16.09°
		1	20	-14.5	
		2.5	63	+16.09	
		2.5	63	+16.09	
		2.5	63	+16.09	
		2.5	63	+16.09	
Ribonic.	Hudson.	2.5	63	-16.09	16.09°
		1	20	-14.5	
		2.5	63	+16.09	
		2.5	63	+16.09	
		2.5	63	+16.09	
		2.5	63	+16.09	

³ Nef, J. U., *Ann. Chem.*, 1914, cdiii, 204.

EXPERIMENTAL.

The phenylhydrazides of gluconic, mannonic, galactonic, allonic, and arabonic acids were prepared in the usual way and were recrystallized out of water. They were obtained in the form of perfectly colorless bright plates.

The idonic, gulonic, talonic, altronic, and ribonic acids were pre-

precipitated out of alcoholic solutions by ether. A slightly gelatinous perfectly white substance settled out, which crystallized out of alcohol in the form of colorless plates. Idonic acid was prepared by the method of Van Ekenstein and Blanksma.⁴

With a few exceptions all readings were made in a 2 dm. tube. Practically all readings were made with an accuracy of $\pm 0.00^\circ$.

⁴ Van Ekenstein, W. A., and Blanksma, J. J., *Rec. trav. chim. Pays-Bas*, 1908, xxvii. 1.

CEREBROSIDES.

III. CONDITIONS FOR HYDROLYSIS OF CEREBROSIDES.

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The conditions of hydrolysis of cerebrosides followed by most investigators remain as they were introduced by Thudichum,¹ the pioneer worker in this field. Thudichum made use of two methods. One consisted in heating the cerebrosides in a 2 per cent aqueous sulfuric acid solution in sealed tubes for a period varying from 310 to 370 hours. In the second method, the substance was decomposed by heating a solution in ethyl alcohol containing sulfuric acid, under a reflux. These methods remain quite serviceable for experiments of a qualitative nature. However, the methods proved imperfect for an investigation into the quantitative relationships of the components of cerebrosides. Indeed, Thudichum never intended them for that purpose. On the other hand subsequent workers who attempted the quantitative analysis of cerebrosides followed Thudichum's directions without having ascertained the degree of their accuracy.

Early in our work on the hydrolysis of cerebrosides we became aware of the fact that the original methods of hydrolysis demanded improvement from the standpoint of convenience and reliability. The original aqueous hydrolysis was carried out for a period of over 300 hours. It is evident that this prolonged heating must bring about a partial decomposition of the galactose. Again, the alcoholic hydrolysis (partial alcoholysis) was found both inconvenient and inaccurate. Thudichum had demonstrated the presence of some intermediate substances among the products of hydrolysis. Furthermore, the mineral acid in the presence of alcohol catalyzes not only the process of splitting the original substances, but also the synthetic processes between alcohol and the cleavage products. Hence the products of reaction on boiling the cerebrosides with an alcoholic solu-

¹ Thudichum, J. L. W., *Physiological Chemistry of the Brain*, London, 1884.

tion of a mineral acid are galactose, ethyl galactoside, fatty acids and their ethyl esters, sphingosine, and mono- and diethyl sphingosine. The separation of these substances is not a simple matter.

Because of this, an attempt was made to find conditions of hydrolysis under which the components of the cerebrosides could be obtained in a fair degree of purity and in a quantity approaching the values required by theory. It was found that two operations were required, one for determining the sugar content, and a second for determining the base and acid. In connection with the sugar estimation the older methods particularly needed improvement. During a long period when the older methods of hydrolysis were followed in our laboratory, a yield of glucose approaching the theoretical requirement was obtained only once. A scrutiny of the results published by Thierfelder² and his coworkers shows that they also encountered in individual samples of cerebrosides greater sugar variations than one should have expected from the practically constant elementary composition of the substances.

Hence we undertook to determine the destructive influence on galactose of mineral acids in concentrations usually employed for hydrolysis of cerebrosides. The details are given in the tables.

On the basis of these tables the following conditions were selected for the hydrolysis of cerebrosides, aiming at the sugar estimation. 1.0 gm. of cerebrosides and 16 cc. of 3 per cent sulfuric acid were heated with shaking in a sealed tube for 12 hours at 105°. The filtrate from the insoluble cake combined with the wash water was used for the sugar estimation. When larger quantities are employed for hydrolysis the proportions remain the same. Under such conditions approximately 90 per cent of the sugar present in the cerebroside is obtained. We were unable to obtain more accurate results. We also wish to remark that when a small quantity of cerebroside is used the sugar cannot be estimated polarimetrically with a sufficient degree of accuracy.

² Loening, H., and Thierfelder, H., *Z. physiol. Chem.*, 1911, lxxiv, 282. Thierfelder, *ibid.*, 1913, lxxxv, 35; 1914, lxxxix, 236. See also Argiris, A., *ibid.*, 1908, lvii, 289.

TABLE I.

Galactose.

1.3 gm. of galactose dissolved in 100 cc. of 10 per cent HCl + C_2H_5OH , 4:1. Portions of 20 or 10 cc. hydrolyzed for various lengths of time.

	Per cent.	Loss.	Per cent loss.
Original solution.....	0.546	0	0
8 hrs.....	0.380	0.166	30.40
12 ".....	0.304	0.242	44.40
24 ".....	0.131	0.415	76.00
48 ".....	0.022	0.524	96.00

II. 3 gm. of galactose dissolved in 100 cc. of 2 per cent HCl.

	Per cent.	Loss.	Per cent loss.
Original solution.....	0.511	0	0
2 hrs.....	0.511	0	0
4 ".....	0.4965	0.0145	2.85
8 ".....	0.4825	0.0285	5.54
24 ".....	0.4590	0.0520	10.23

III. 3 gm. of galactose dissolved in 100 cc. of 4 per cent H_2SO_4 .

	Per cent.	Loss.	Per cent loss.
Original solution.....	0.5355	0	0
2 hrs.....	0.5355	0	0
4 ".....	0.5105	0.0250	4.66
8 ".....	0.5070	0.0285	5.34
24 ".....	0.4820	0.0535	10.00

IV. 1 gm. of galactose and 40 cc. of alcohol containing 7 per cent H_2SO_4 boiled for 4 hours and then hydrolyzed with 3 per cent H_2SO_4 for various lengths of time.

	Sugar.
	gm.
Solution before hydrolysis.....	0.0000
1 hr. hydrolysis.....	0.6000
3 " ".....	0.8925
4 " ".....	0.6950

TABLE I—*Concluded.**Phrenosin.*

I. 2 gm. of phrenosin boiled with 40 cc. of 10 per cent HCl + 95 per cent alcohol 4: 1 for varying lengths of time.

Amount of phrenosin.	Time of boiling.	Sugar.	
<i>gm.</i>	<i>hrs.</i>	<i>gm.</i>	<i>per cent</i>
2.0000	8	0.205	10.25
1.9946	12	0.084	0.42
1.9974	24	0.031	0.15
2.0004	48	0.000	0.00

II. 3 gm. of phrenosin boiled with absolute alcohol and 40 cc. of 7 per cent H₂SO₄ for 4 hours, and then hydrolyzed with 4.4 per cent H₂SO₄.

Found.....11.8 per cent.

III. 3 gm. of phrenosin hydrolyzed with 50 cc. of 3 per cent H₂SO₄ + 5 cc. of alcohol.

	Sugar,
<i>hrs.</i>	<i>per cent</i>
16.....	17.2
24.....	16.3

IV. 1 gm. of phrenosin heated in a sealed tube with 16 cc. of 3 per cent H₂SO₄ at 100° for various lengths of time.

	Sugar.
<i>hrs.</i>	<i>per cent</i>
2.....	6.8
4.....	15.48
8.....	16.32
11.....	17.98
14.....	18.25
16.....	17.68
24.....	16.60

Analysed according to Rosenheim's method (*Biochem. J.*, 1916, x, 146), 11.56 per cent.

TABLE II.

Hydrolysis in Sealed Tube for 12 Hours at 105°.

Sample No.	Sugar.
	<i>per cent</i>
60 C	18.8
61 C	18.6
62 C	21.0
272 C	19.2
366 C	18.8
514 C	18.72
Theory.	21.8

For the purpose of estimating the base and the fatty acids the following conditions were found satisfactory: 1 gm. of cerebroside was heated with 3 per cent sulfuric acid in a sealed tube with shaking for 24 hours at 105°. After cooling the reaction product was filtered. The insoluble cake was washed with distilled water on the filter, then pressed between filter paper to remove adhering water. The filter paper with the substance was transferred to boiling methyl alcohol to which a few drops of phenolphthalein were added. This was followed by the addition of a hot saturated solution of barium hydroxide in methyl alcohol until the reaction was strongly alkaline. Acetone was then added as long as a precipitate formed. When all the barium hydroxide and the barium salts are precipitated the pink solution turns colorless. The solution contains the base, and the precipitate consists of the barium salts of the fatty acids. For the purpose of purification the fatty acids were liberated with hydrochloric acid, and redissolved with acetone. This operation was repeated three times. The soaps were suspended in water, decomposed with hydrochloric acid, a little benzene was added, and the flask was placed on a water bath until the benzene was evaporated. On cooling, the acids floated on the surface of the liquid. They were then filtered and dissolved in hot acetone to remove the inorganic salts. The acetone solution on evaporation left a dry residue, ready for analysis. All the mother liquors were added to the original solution containing the base. The combined solution was evaporated nearly to dryness and extracted with hot acetone. The operation was repeated until the final

residue was completely soluble in acetone. All the insoluble residues were added to the soaps. The acetone solution of the bases was evaporated and dried to constant weight.

This method has been tried out for 4 years and has always given results which were as accurate as could be expected for substances of that nature. Table III contains the results of only a few of the analyses carried out by this method.

TABLE III.

Sample No.	Base.	Fatty acid.
	<i>per cent</i>	<i>per cent</i>
60 C	32.7	43.0
61 C	31.0	48.6
62 C	30.0	42.8
366 C	35.8	48.52
514 C	32.1	46.7
Theory.	34.5	48.1

The results of the elementary composition of the individual fractions were as follows.

The acids were analyzed directly after removing the acetone, if they were free from inorganic impurities. Otherwise, they were dissolved in ether and freed from impurities by shaking with dilute hydrochloric acid and then with water. The ethereal solution was then freed from ether and the residue analyzed.

60 C 0.1000 gm. substance gave 0.2890 gm. CO_2 and 0.1150 gm. H_2O .

61 C 0.1040 " " " 0.2888 " " " 0.1162 " "

62 C 0.1050 " " " 0.2894 " " " 0.1162 " "

366 C 0.1055 " " " 0.2886 " " " 0.1142 " "

514 C 0.1070 " " " 0.3018 " " " 0.1200 " "

	Calculated for:		Found:				
	$\text{C}_{25}\text{H}_{45}\text{O}_2$	$\text{C}_{25}\text{H}_{40}\text{O}_2$	60 C	61 C	62 C	366 C	514 C
C.....	78.2	75.33	75.10	75.74	75.16	74.60	77.00
H.....	13.2	12.50	12.25	12.50	12.38	12.10	13.03

The base was analyzed in form of the sulfate. In order to obtain this, the fraction was dissolved in alcohol, and an alcoholic solution

of sulfuric acid was added until the mixture reacted markedly acid to litmus. The mixture was allowed to stand 15 to 24 hours at 0°C., filtered, and washed, first with acetone and then with ether, and dried under diminished pressure.

60 C	0.1064 gm.	substance gave 0.0226 gm. CO ₂ and 0.0962 gm. H ₂ O.
61 C	0.0998 "	" " " 0.2334 " " " 0.0998 " "
62 C	0.0984 "	" " " 0.2264 " " " 0.0976 " "
514 C	0.1012 "	" " " 0.2252 " " " 0.0980 " "

	Calculated for (C ₁₇ H ₃₅ NO ₂) ₂ H ₂ SO ₄ :	Found:			
		60 C	61 C	62 C	514 C
C.....	61.08	60.76	64.42	62.87	61.29
H.....	10.78	10.00	11.28	11.12	10.95

The analytical figures for the sulfates deviate considerably in some instances from the theory. This however, is not due to the presence of impurities but occurred when an insufficient amount of sulfuric acid was added to form the neutral salt. One or two recrystallizations generally suffice to obtain a pure product. With more experience in the work analytically pure samples are obtainable without recrystallization.

50 gm. of material are a convenient quantity for analysis. Very satisfactory results were obtained when 1.5 gm. were hydrolyzed. When the analysis of the fatty acid fraction obtained by this method shows the presence of both lignoceric and cerebronic acid, and when the identification of each one is desired it is advantageous to resort to a separate hydrolysis for each acid. For the isolation of cerebronic acid, the procedure is as follows.

10 gm. of the cerebrosides and 150 cc. of a 10 per cent hydrochloric acid solution to which 30 cc. of 98 per cent ethyl alcohol are added, are heated for 24 hours in a flask provided with a reflux condensor and a mechanical stirrer. On cooling, a solid cake is formed on the surface of the liquid. This is dissolved in hot methyl alcohol. A solution of barium hydroxide is then added and the soaps are purified exactly in the manner described above.

The acid obtained in this manner generally analyzes as follows:

A sample of cerebroside (94 C) with $[\alpha]_D^{20} = 0.00^\circ$:

0.1144 gm. substance gave 0.3176 gm. CO_2 and 0.1257 gm. H_2O .

C = 75.71; H = 12.30.

A sample of cerebroside (221 B) with $[\alpha]_D^{20} = -2.0^\circ$:

0.1166 gm. substance gave 0.3248 gm. CO_2 and 0.1317 gm. H_2O .

C = 75.85; H = 12.64.

From the optical rotation of the substances it is evident that they contained cerasin. On the other hand samples of the same nature yield pure lignoceric acid when decomposed by means of alcohol containing sulfuric acid. The details are as follows.

10.0 gm. of cerebroside are taken up in 100 cc. of 99.5 per cent alcohol containing 10.0 gm. of sulfuric acid and heated in a boiling water bath for 8 hours. The solution is allowed to cool over night at 25°C . Ethyl lignocerate settles out in the form of bright scales. These are removed by filtration. The precipitate may be dissolved in ether, the solution dried over potassium carbonate, filtered, and analyzed in the form of the ester. It also may be converted into the free acid.

Treated in this manner a sample (24 B) with $[\alpha]_D^{20} = 0.00^\circ$ yielded an acid of the following composition:

0.1183 gm. substance gave 0.3388 gm. CO_2 and 0.1139 gm. H_2O .

C = 78.10; H = 13.15.

CEREBROSIDES.

IV. CERASIN.

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Thudichum¹ was the first to assume the existence of more than one cerebroside in brain tissue. By fractionation from alcohol he succeeded in isolating from the crude cerebroside mixture two substances. One he considered an individual glucoside, which he named phrenosin. The other substance he regarded as a second cerebroside, cerasin, still containing some phrenosin. Apparently for lack of material, Thudichum did not accomplish the task of preparing a pure sample of cerasin, nor did he furnish any information regarding the chemical differences of the two cerebroside.

Subsequent workers have rediscovered phrenosin² and, in a general way, have substantiated Thudichum's claim for its individuality.

Regarding the second cerebroside, cerasin, no essential progress was made for many years. The substances, phrenosin and cerasin, were distinguished by their solubilities and their physical appearance. However, in 1913, a chemical distinction between phrenosin and cerasin was twice discovered, nearly simultaneously. It was found that from the so called cerasin, a fatty acid of the composition, $C_{24}H_{48}O_2$, could be isolated, whereas pure phrenosin contained only cerebronic acid, $C_{25}H_{50}O_3$. In this respect it is worthy of note that Thudichum foresaw the possibility of the two cerebroside differing only by the nature of their fatty acids. The new acid was recognized independently by Levene³ and by Rosenheim⁴ as lignoceric acid. There still seems, however, to exist a lack of convincing evidence that cerasin has ever

¹ Thudichum, J. L. W., *Physiological Chemistry of the Brain*, London, 1884.

² Loening, H., and Thierfelder, H., *Z. physiol. Chem.*, 1911, lxxiv, 282; 1912, lxxvii, 202. Thierfelder, *Z. physiol. Chem.*, 1913, lxxxv, 35; 1914, lxxxix, 236.

³ Levene, P. A., *J. Biol. Chem.*, 1913, xv, 359.

⁴ Rosenheim, O., *Tr. Int. Cong. Med.*, 1913, ii, 626; *Biochem. J.*, 1916, x, 142.

been obtained free from phrenosin. It is true, on the basis of a new test applied to the study of cerebrosides, namely, the selenite plate test, Rosenheim claimed to have isolated pure cerasin. However, comparing the optical activity of the various samples obtained by Rosenheim with those prepared by us, we feel certain that our material was of the same degree of purity as that of the previous author. The samples of Rosenheim varied between $[\alpha]_D = -2.50^\circ$ and -3.71° , whereas the $[\alpha]_D$ of our samples varied between -2.25° and -3.45° .

The first samples of cerasin of that nature were obtained by us in 1913. The results were not published at that time for the reason that we hoped to obtain purer material by means of the older methods of fractional crystallization, or fractional extraction by means of organic solvents. We soon became convinced, however, that the results of the operations were variable, even when the conditions of purification seemed constant. Evidently some unknown factors played a part and since the factors remained unknown, they could not be controlled. Hence the method of fractionation was abandoned and a search was made for such derivatives of phrenosin and cerasin, which would possess properties suitable for the separation of one from the other.

With this aim in view the properties of the acetyl, benzoyl, cinnamoyl, and *p*-nitrobenzoyl derivatives were investigated. Benzoylation was selected as the simplest and best method for the separation of the two compounds. A convenient way of benzoylation was found in the use of pyridine as a solvent, a method first introduced by Einhorn. The saponification of the benzoyl derivative is best accomplished by means of sodium methylate. These are the two essential points of the process, the details of which are given in the experimental part.

It should be noted here that Rosenheim⁵ mentioned the possibility of accomplishing the separation of the two cerebrosides through benzoylation. Thierfelder⁶ later suggested the use of the acetyl derivatives.

It must, however, be borne in mind that a satisfactory result is obtained only when the starting material contains a moderate propor-

⁵ Rosenheim, *Biochem. J.*, 1914, viii, 110.

⁶ Thierfelder, *Z. physiol. Chem.*, 1914, lxxxix, 248.

tion of phrenosin; namely, a mixture with $[\alpha]_D = 0.0^\circ$ in pyridine. Hence, the complete process is composed of an initial step consisting of fractional precipitation or fractional extraction, and a second step, consisting of separation by means of benzylation. As a result of this second step a product is obtained with $[\alpha]_D = -2.50$ to -3.50° .

This levorotation is nearly that obtained on previous occasions by Rosenheim in 1916 and by us in 1913. As in 1913, we are convinced now also that this material is not yet free from phrenosin. This view is based on the fact that, on hydrolysis with aqueous sulfuric acid under conditions described by Levene and Meyer,⁷ cerasin, with the highest levorotation, yields a mixture of fatty acids containing about 77.0 per cent carbon. Furthermore, on hydrolysis in a solution of 10 per cent hydrochloric acid, containing 15 per cent alcohol, one obtains either pure cerebronic acid, or a mixture of cerebronic and lignoceric acids, whereas, by using alcohol containing 8 to 10 per cent sulfuric acid (by weight), one obtains pure lignoceric acid. Hence, it is evident that one may be led into an error if he bases his conclusion on the latter mode of cleavage (alcoholysis). The find of Rosenheim may be explained by the fact that he employed his method of hydrolysis.

Thus, a complete separation of cerasin from phrenosin has not as yet been accomplished. The present results are reported at this time because of lack of confidence in a speedy solution of the problem. For the present we are engaged in the preparation of large quantities of material with a rotation of approximately -3.0° .

In a way the topic discussed here may appear to possess a merely academic interest, since the existence of the two cerebroside is quite certain. The only alternative hypothesis would be the existence of one simple cerebroside, phrenosin, while cerasin might be a dicerebroside. This view is contradicted by the molecular weight determinations by Kossel and Freytag,⁸ who found a mean value of 986, using glacial acetic acid as the solvent, and by Rosenheim,⁹ who found a mean of 763, using Barger's microscopic method, based on vapor

⁷ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 627.

⁸ Kossel, A., and Freytag, F., *Z. physiol. Chem.*, 1893, xvii, 445.

⁹ Rosenheim, *Biochem. J.*, 1916, x, 153.

pressure, with pyridine as the solvent. However, the final and absolute proof of the existence of a substance is its isolation.

EXPERIMENTAL.

Preparation of the Mixture of Cerebrosides.

The crude cerebrosides used in this work were prepared essentially by the method of Rosenheim.¹⁰ The principal feature of this method consists in dissolving the "white matter" (mixture of cerebrosides and sphingomyelin) in pyridine. The difference in the procedure was that the "white matter" was heated in commercial pyridine, until the solution was complete. This solution was then allowed to stand over night at room temperature. The filtrate contained the cerebrosides. This was concentrated to a small volume and poured into alcohol. The precipitate thus obtained was dissolved in hot 90 per cent alcohol, filtered from insoluble oily material, and again allowed to cool. The precipitate was repeatedly extracted with ether. The final insoluble produce was composed of the mixed cerebrosides.

Separation into Phrenosin and Cerasin Fractions.

The separation into phrenosin and cerasin fractions was accomplished by means of acetone. Two fractions were obtained, one practically insoluble in boiling acetone, the other soluble. Each of these fractions was a mixture of two cerebrosides. However, the first (insoluble) contained a very high proportion of phrenosin while in the second (soluble) cerasin was predominating.

The procedure used was as follows: 100 gm. of the cerebrosides were dissolved in 50 cc. of boiling 95 per cent ethyl alcohol and to this solution 3 liters of boiling acetone were added. A precipitate formed immediately. This was removed by filtering with suction, care being taken to warm the funnel before filtration. The insoluble part yields practically pure phrenosin when redissolved in boiling alcohol and then allowed to crystallize in a thermostat at 42°C.

The acetone-solution fraction was concentrated to a small volume and the cerebrosides were allowed to separate out. The material ob-

¹⁰ Rosenheim, *Biochem. J.*, 1914, viii, 110; 1916. x, 142.

tained in this manner was further fractionated from methyl alcohol. For this purpose it was dissolved in methyl alcohol and then allowed to stand at 40° . This gave the fraction I a_1 . The filtrate from I a_1 was then allowed to cool to room temperature when a second precipitate settled out. This was designated I a_2 . On cooling to 0°C . a third fraction, I a_3 , was obtained.

The further purification of each of these fractions was laborious, various modifications of Rosenheim's original method being tried, and the results were variable. These fractions have no interest since the introduction of the benzoylation process. In order to obtain material for this process, the crude cerebrosides may be dissolved in a hot solution of one part ethyl alcohol and three parts chloroform (by volume). On standing at room temperature a precipitate forms, which is removed by filtration. The mother liquor is concentrated and either allowed to crystallize or is poured into acetone. Usually this material has a rotation of about 0.0° (in pyridine). If it is higher ($+0.02$ to $+0.04^{\circ}$) the operation is repeated. In some cases it was found that the mother liquor of the crystallization of the crude cerebrosides from ten parts of ethyl alcohol also gave a product with a rotation of $[\alpha]_D = 0.0^{\circ}$.

Further Separation of Cerasin from Phrenosin by Benzoylation.

As mentioned above, Rosenheim⁵ first suggested the possibility of a separation of phrenosin and cerasin by means of the benzoyl derivatives. We have also examined the acetyl derivatives as a method of separation but find it unsatisfactory, especially when compared with the benzoyl method.

The acetyl derivative of the material with a rotation of about -0.02° was prepared as described below and separated into two fractions, soluble and insoluble in methyl alcohol at 0° . The two fractions were then saponified with sodium methylate, and gave products with $[\alpha]_D$ of -0.02° and -0.02° . This indicates that no separation has been effected.

The benzoyl derivative was prepared in pyridine as described on page 158. The product, after removing the ether on the water bath, is taken up in methyl alcohol and cooled to 0° over night. The

supernatant liquid is decanted, concentrated to a small volume, poured into a solution of sodium methylate in methyl alcohol, and the mixture heated 2 hours. Usually the cerebroside separates out during the heating. After cooling, the precipitate is filtered off, washed thoroughly with methyl alcohol and then with acetone, and crystallized from methyl alcohol until ash-free. It is usually necessary to decolorize the solution with animal charcoal in order to obtain a colorless product. Such material showed values of $[\alpha]_D$ varying from -2.2 to -3.2° in pyridine.

$$[\alpha]_D^{30} = \frac{7.4810 \times -0.08^\circ}{0.4180 \times 0.5} = -2.86^\circ$$

$$[\alpha]_D^{20} = \frac{7.9450 \times -0.07^\circ}{0.5028 \times 0.5} = -2.25^\circ$$

$$[\alpha]_D^{20} = \frac{6.1186 \times -0.08^\circ}{0.306 \times 0.5} = -3.2^\circ$$

Since the first purification through the benzoyl derivative was so successful, it was thought that the repetition of the process would yield a cerasin with a still higher rotation. A product with $[\alpha]_D^{20} = -2.86^\circ$ was therefore benzoylated and the methyl alcohol mother liquor saponified as above. The cerebroside, twice crystallized from methyl alcohol, showed a value for $[\alpha]_D^{20}$ of -2.98° .

$$[\alpha]_D^{20} = \frac{5.101 \times -0.10^\circ}{0.3440 \times 0.5} = -2.98^\circ$$

This indicates little or no purification. No better results were obtained with the acetyl derivative. The material before acetylation had a value of $[\alpha]_D$ of -2.30° , and the product after saponification of the acetyl derivative showed $[\alpha]_D = -2.34^\circ$.

Hydrolysis of Cerasin Obtained by Fractionation.

The fraction most soluble in methyl alcohol was further fractionated out of pyridine and chloroform, which gave a product with a rotation of -2.24° .

$$[\alpha]_D^{20} = \frac{5.281 \times -0.17^\circ}{0.400 \times 1} = -2.24^\circ$$

30 gm. of this material were hydrolyzed with 450 cc. of 10 per cent hydrochloric acid and 75 cc of 95 per cent alcohol. The acids obtained from this hydrolysis, worked up in the usual way, had the following composition:

0.1167 gm. substance gave 0.3293 gm. CO_2 and 0.1324 gm. H_2O .

	Calculated for		Found:
	$\text{C}_{21}\text{H}_{42}\text{O}_2$:	$\text{C}_{23}\text{H}_{46}\text{O}_2$:	
C.....	78.20	75.33	76.95
H.....	13.16	12.50	12.70

The fraction I a_2 (page 153) was extracted with dilute acetone (one part water to ten parts acetone). The insoluble part had a rotation of -3.45° .

$$[\alpha]_D^{20} = \frac{5.2834 \times -0.28^\circ}{0.400 \times 1} = -3.45^\circ$$

20 gm. of this material were hydrolyzed by boiling for 6 hours with 150 cc. of 98 per cent alcohol containing 7.5 cc. of sulfuric acid. On standing over night at room temperature, scales of ethyl lignocerate separated out.

0.1129 gm. substance gave 0.3240 gm. CO_2 and 0.1318 gm. H_2O .

	Calculated for	Found:
	$\text{C}_{23}\text{H}_{46}\text{O}_2$:	
C.....	78.8	78.26
H.....	13.10	13.16

15 gm. of the same sample were then hydrolyzed with 150 cc. of 10 per cent hydrochloric acid and 15 cc. of 95 per cent alcohol. The fatty acids were obtained through the barium salts. The acids were then transferred into the ethyl ester by boiling in a solution of 100 cc. of 98 per cent alcohol containing 5 gm. sulfuric acid for 6 hours. On standing, scales separated out, with the following composition.

0.1150 gm. substance gave 0.3264 gm. CO_2 and 0.1306 gm. H_2O .

	Calculated for	Found:
	$\text{C}_{23}\text{H}_{46}\text{O}_2$:	
C.....	78.80	77.40
H.....	13.10	12.71

Hydrolysis of Cerasin Obtained by the Benzoylation Process.

3 gm. of the material with $[\alpha]_D^{20} = -3.25^\circ$ were heated in a sealed tube with 50 cc. of 3 per cent sulfuric acid at $100-105^\circ$ for 18 hours. The acids were isolated through the barium salts and had the following composition.

0.1044 gm. substance gave 0.2940 gm. CO_2 and 0.1152 gm. H_2O .

	Calculated for		Found:
	$\text{C}_{23}\text{H}_{43}\text{O}_2$:	$\text{C}_{25}\text{H}_{49}\text{O}_3$:	
C.....	78.20	75.33	76.80
H.....	13.16	12.50	12.35

Acetyl Phrenosin.

Acetyl phrenosin was first prepared by Thierfelder.⁶ 20 gm. of phrenosin, 20 gm. of fused sodium acetate, and 200 cc. of acetic anhydride were heated to gentle boiling for $\frac{1}{2}$ hour. The excess of acetic anhydride was removed by distillation in vacuum and the semi-solid residue dissolved in ether and water. The ether solution of the acetyl derivative was washed with water, with dilute alkali, and again with water, dried over sodium sulfate, and the ether concentrated on the water bath. The residue was taken up in hot dry methyl alcohol, in which it is very soluble, and from which it separates in a granular condition upon cooling. After the second crystallization from methyl alcohol, the yield was 14 gm. of a product with $[\alpha]_D = -10.4^\circ$. The third crystallization gave a product with $[\alpha]_D = -11.07^\circ$, which was not changed on further purification.

Acetyl phrenosin melts somewhat unsharply at $41-43^\circ$. Thierfelder gives the melting point as $39-41^\circ$ and the rotation as -3° . Our product, dissolved in a mixture of equal parts of chloroform and methyl alcohol (by volume) showed the following rotation:

$$[\alpha]_D^{20} = \frac{7.0708 \times -0.39^\circ}{0.4980 \times 0.5} = -11.07^\circ$$

$$[\alpha]_D^{20} = \frac{6.5570 \times -0.43^\circ}{0.5066 \times 0.5} = -11.08^\circ$$

0.1052 gm. substance gave 0.2568 gm. CO₂ and 0.0892 gm. H₂O.

0.5000 gm. substance neutralized 4.35 cc. 0.1 N HCl.

0.500 gm. substance, used for an acetyl determination, required 28 cc. 0.1 N HCl, and in a second experiment, 28.2 cc.

0.673 gm. substance, in 22.56 gm. chloroform produced a rise in boiling point of 0.108°.

0.943 gm. substance, in 22.56 gm. chloroform, produced a rise in boiling point of 0.158°.

	Calculated for hexacetylphrenosin C ₆₀ H ₁₀₈ NO ₁₈ :		Found:
C.....	66.68		66.57
H.....	9.80		9.49
N.....	1.29		1.22
Ac.....	23.8	24.08	24.24
Mol. wt.....	1079	1003	960

One experiment was made to determine whether the same derivative could be prepared by the use of acetyl chloride. 20 gm. of phrenosin were dissolved in 150 cc. of pyridine, the solution was cooled to 0°C., 16 cc. of acetyl chloride were added in portions, and the mixture was allowed to stand 2 days at 0°C. The pyridine hydrochloride was filtered off, the solution washed with water, dilute hydrochloric acid, and then with water, dried with sodium sulfate, and concentrated. The residue was crystallized repeatedly from methyl alcohol. After the fourth crystallization, it had a rotation of -8.41°, and melted at about 40°.

$$[\alpha]_D^{20} = \frac{6.1218 \times -0.33^\circ}{0.4794 \times 0.5} = -8.41^\circ$$

The analysis indicates that it is probably a mixture of acetyl derivatives. It was not purified further.

0.1008 gm. substance gave 0.2484 gm. CO₂ and 0.0896 gm. H₂O.

	Calculated for Hexacetyl- phrenosin C ₆₀ H ₁₀₈ NO ₁₈ :	Triacetyl- phrenosin C ₅₄ H ₉₉ NO ₁₂ :	Found:
C.....	66.68	67.94	67.22
H.....	9.80	10.46	9.96

Acetyl Cerasin.

20 gm. of a cerasin with $[\alpha]_D^{20} = -2.8^\circ$, 20 gm. of sodium acetate, and 200 cc. of acetic anhydride were boiled under a reflux for $\frac{1}{2}$ hour.

The reaction product was worked up as described above, and the product then twice crystallized from methyl alcohol. This material did not show a change in rotation upon further purification from methyl alcohol. It is slightly more insoluble in methyl alcohol than is acetyl phrenosin, as stated by Thierfelder.⁶ Acetyl cerasin melts at 54–56°, and in a mixture of equal parts of chloroform and methyl alcohol gave the rotation:

$$[\alpha]_D^{20} = \frac{5.9310 \times -0.60^\circ}{0.4324 \times 0.5} = -16.46^\circ$$

0.1035 gm. substance gave 0.2576 gm. CO₂ and 0.0932 gm. H₂O.

0.500 gm. substance neutralized 4.4 cc. 0.1 N HCl.

	Calculated for pentacetylcerasin C ₅₇ H ₁₀₁ NO ₁₃ :	Found:
C.....	67.87	67.75
H.....	10.10	10.00
N.....	1.40	1.23

As stated on page 153, the acetyl derivatives are not a suitable means of separating phrenosin and cerasin.

Benzoylphrenosin.

10 gm. of practically pure phrenosin were dissolved in 100 cc. of pyridine, cooled to room temperature, and treated with 12 cc. of benzoyl chloride. The reaction mixture was cooled under the water tap and then allowed to stand at 0°C. over night. The next day the pyridine hydrochloride was filtered off, the solution concentrated in vacuum at 50°C., and the resulting oily liquid poured, with stirring, into a large volume of about 2 per cent sodium hydroxide solution. The benzoylphrenosin separated as an oil on the sides and bottom of the dish. After washing with water and hydrochloric acid, the material was taken up in ether and dried with sodium sulfate. An alternative procedure is to dissolve the pyridine containing oily material in ether, then shake with dilute acid and alkali, then with water, and dry this solution. After removal of the ether the product is taken up in hot methyl alcohol. From a concentrated solution, the benzoyl derivative separates as an oil, which solidifies on cooling.

From a sufficiently dilute solution it separates as colorless nearly crystalline material, which is easily filtered. It may also be obtained in a granular form from ethyl alcohol. It is easily soluble in acetone, benzene, petroleum ether, chloroform, acetic acid, and pyridine.

In an experiment in which 80 gm. of phrenosin were used, the benzoyl derivative was purified by crystallization from methyl alcohol, then from acetone (in which about 50 per cent of the material was lost), and then from methyl alcohol until the rotation was constant. The melting point as observed on this material was fairly sharp at 65–66°.

In methyl alcohol and chloroform the rotation is:

$$\begin{aligned}\text{I. } [\alpha]_D^{20} &= \frac{7.1484 \times + 0.75^\circ}{0.5062 \times 0.5} = + 21.1^\circ \\ \text{II. } [\alpha]_D^{20} &= \frac{6.1518 \times + 0.92^\circ}{0.5320 \times 0.5} = + 21.27^\circ \\ \text{III. } [\alpha]_D^{20} &= \frac{6.1060 \times + 0.91^\circ}{0.5240 \times 0.5} = + 21.20^\circ\end{aligned}$$

Sample I was obtained from material which contained at least 75 per cent cerasin. Samples II and III were obtained from material considered nearly pure phrenosin. The analysis and molecular weight indicate that this material of constant rotation was probably a tribenzoylphrenosin.

0.1004 gm. substance gave 0.2704 gm. CO₂ and 0.0792 gm. H₂O.

0.1028 " " " 0.2784 " " " 0.0866 " "

0.1000 " " " 0.2707 " " " 0.0776 " "

0.5000 " " neutralized 4.40 cc. 0.1 N HCl.

0.5000 " " " 4.10 " 0.1 "

0.500 " " used for a benzoyl determination required 11.6 cc. 0.1 N NaOH, and in a second experiment 11.9 cc.

0.255 " " in 23.21 gm. chloroform raised the boiling point 0.026°.

0.682 " " as above, raised the boiling point 0.108°.

1.008 " " " " " " " " 0.160°.

	Calculated for tribenzoylphrenosin C ₆₃ H ₁₀₅ NO ₁₂		Found:	
C.....	72.65	73.45	73.89	73.81
H.....	9.28	8.83	9.43	8.69
N.....	1.23		1.23	1.12
C ₆ H ₅ CO.....	25.32		24.36	24.99
Mol. wt.....	1,139	1,037	1,066	1,092
Average.....			1,065	

An attempt was then made to prepare a hexabenzoyl derivative. 40 gm. of the above mentioned material were dissolved in 300 cc. of pyridine and treated with 400 cc. of benzoyl chloride. After working up as usual, the product was taken up in methyl alcohol. An oil formed which would not solidify even after standing several weeks. Since the material with constant rotation served our need the experiment was discarded.

Phrenosin from Benzoylphrenosin.

About 10 gm. of sodium were dissolved in 300 cc. of methyl alcohol and 20 gm. of benzoylphrenosin added with shaking (the benzoylphrenosin may also be added in acetone solution). A white precipitate gradually forms upon boiling (immediately, if an acetone solution is used). After boiling for 2 hours, the reaction product is cooled in the ice box, the precipitate filtered off, recrystallized from methyl alcohol, then from glacial acetic acid, and finally from a mixture of equal parts of methyl and ethyl alcohols.

The phrenosin crystallizes practically quantitatively, for upon concentration of the mother liquor only sodium benzoate was obtained. The rotation of this material in pyridine corresponds well with that found for the phrenosin obtained by fractionation of the top fraction of the cerebroside mixture. Rosenheim gives $+3.78^\circ$ and $+3.70^\circ$.

In pyridine the rotation was:

$$[\alpha]_D^{20} = \frac{+8.0000 \times 0.12^\circ}{0.5232 \times 0.5} = +3.67^\circ$$

Cinnamoylphrenosin.

10 gm. of phrenosin were dissolved in 100 cc. of pyridine and 15 gm. of cinnamoyl chloride added. After standing over night at 0° , the filtered solution was concentrated and worked up as described above. The resulting product was purified by crystallization from methyl or ethyl alcohol, or from acetone. Twice recrystallized from methyl alcohol, and once from acetone, the substance analyzed as a tricinnamoylphrenosin. Tricinnamoylphrenosin is slightly less soluble in organic solvents than the benzoyl derivative. It melts at $69-70^\circ$.

$$[\alpha]_D^{20} = \frac{7.2060 \times 0.75^\circ}{0.4976 \times 85} = + 21.72^\circ$$

0.1028 gm. substance gave 0.2718 gm. CO₂ and 0.0868 gm. H₂O.

0.1016 " " " 0.2732 " " " 0.0870 " "

0.500 " " neutralized 4.3 cc. 0.1 N HCl.

	Calculated for tricinnamoylphrenosin C ₇₅ H ₁₁₁ NO ₁₅ :	Found:	
C.....	73.89	73.22	73.33
H.....	9.18	9.45	9.58
N.....	1.15	1.20	

p-Nitrobenzoylphrenosin.

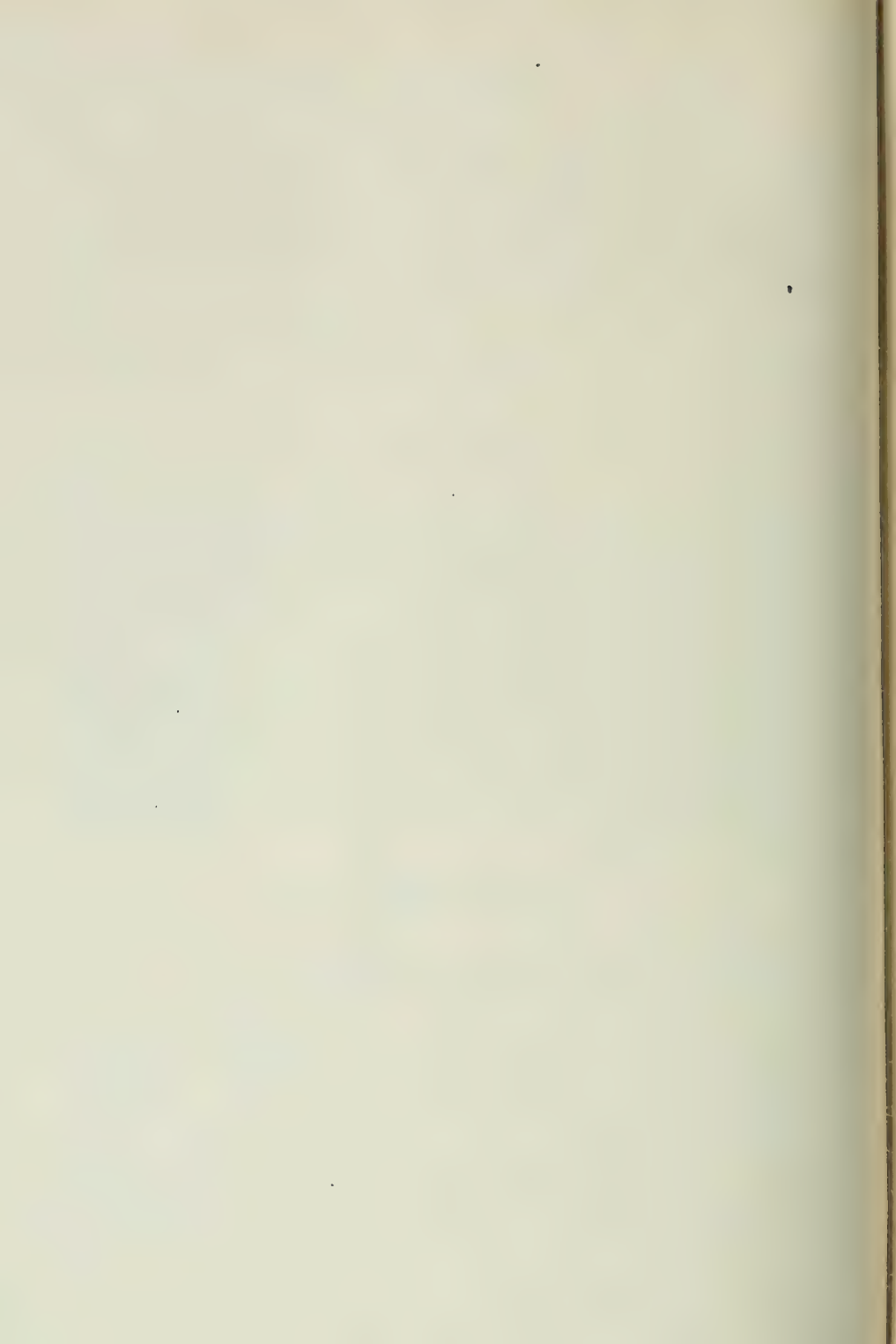
20 gm. of phrenosin, dissolved in 200 cc. of pyridine, were treated with 32 gm. of *p*-nitrobenzoyl chloride, and the mixture was allowed to stand 24 hours at 0°. The reaction product was worked up as usual. The residue from the ether was extracted with hot acetone, the acetone removed on the steam bath, and the product extracted with boiling methyl alcohol. On cooling, the larger part of the nitrobenzoate separated as an oil. This was again extracted with boiling methyl alcohol, and the resulting cake twice crystallized from a large volume of methyl alcohol. *p*-Nitrobenzoylphrenosin is very soluble in acetone, and melts at 94–96°. Analyses indicates that tri-*p*-nitrobenzoylphrenosin is formed. The rotation in chloroform and methyl alcohol was:

$$[\alpha]_D^{20} = \frac{8.6510 \times 0.35^\circ}{0.4970 \times 0.50} = + 12.18^\circ$$

0.1058 gm. substance gave 0.2542 gm. CO₂ and 0.0810 gm. H₂O.

0.1000 " " " 4.00 cc. N gas at 22°C. and 767 mm.

	Calculated for C ₆₉ H ₁₀₂ N ₄ O ₂₃ :	Found:	
C.....	65.00	65.52	
H.....	8.06	8.57	
N.....	4.40	4.67	



CEREBROSIDES.

V. CEREBROSIDES OF THE KIDNEY, LIVER, AND EGG YOLK.

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With the progress of our knowledge of the lipoids of the various organs of the animal body there comes a conviction that there does not exist much variation in the chemical composition of the individual lipoids with the variation of the organs or tissues from which they originate. Up to the present this has been found true in regard to cephalin¹ and sphingomyelin.² The present note contains data regarding the cerebrosides obtained from the same sources. The cerebrosides are found in organs other than the nervous system in very small proportions. Because of this the purification is associated with many difficulties. Furthermore, it is nearly impossible to obtain sufficiently large quantities of material for an exhaustive analysis.

For the present, one is satisfied to be able to show the presence of cerebrosides in several tissues—in the egg yolk, in the liver, and in the kidney—and to be able to state that the composition of these cerebrosides seems to be identical with that of the cerebrosides obtained from the nerve tissue. They contain the same sugar, galactose, the same base, sphingosine, and the same fatty acids, lignoceric and cerebronic.

It is not certain whether the two cerebrosides, phrenosin and cerasin, occur in the same proportions in nerve tissue and in the various other organs. The same remark applies regarding every other individual lipid. It is possible that the proportions do vary with the variation of the organ.

Historically it must be mentioned that the first evidence of existence of cerebrosides in other organs than nervous tissue was furnished

¹ Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 111.

² Levene, *J. Biol. Chem.*, 1916, xxiv, 69.

by Hoppe-Seyler,³ and later by Kossel and Freytag,⁴ who discovered the presence of cerebrosides in pus cells. Recently Rosenheim and MacLean⁵ showed, by the isolation of lignoceric acid and sphingosine from the so called carnaubon of Dunham,⁶ that the kidney probably contained cerebrosides, but these were not isolated in a pure state.

The following table gives the composition and optical activity of the various cerebrosides thus far studied.

	Source.	C	H	N	$[\alpha]_D^{20}$
Phrenosin.....	Brain.	69.51	11.37	1.69	9.6* (3.6)**
Cerasin.....	"	69.53	11.70	1.68	-3.25**
Cerebrosides.....	Kidney.	69.60	11.30	1.68	+14.00*
".....	Liver.	68.36	11.23	1.80	
".....	Egg yolk.	69.32	11.19	1.89	+4.40*
Theory for phrenosin.....		69.65	11.23	1.70	
" " cerasin.....		71.20	11.33	1.73	

* In chloroform and methyl alcohol.

** In pyridine.

EXPERIMENTAL.

Kidney Cerebrosides.

The preparation of the cerebrosides from the kidney was accomplished by following the general plan outlined in the previous communication.⁷ However, in place of pure cerebrosides which are obtained from nerve tissue by this process, the material prepared from the crude kidney "white matter" contained a considerable proportion of neutral fat, the removal of which was found very troublesome. A great aid for the purpose of purification of this material was found in the use of methyl ethyl ketone. By repeated crystallization from this reagent of the crude cerebrosides a substance was finally obtained which had all the properties of the mixed cerebrosides. This mate-

³ Hoppe-Seyler, F., *Med.-Chem. Untersuch.*, 1866-71, 486.

⁴ Kossel, A., and Freytag, F., *Z. physiol. Chem.*, 1893, xvii, 452.

⁵ Rosenheim, O., and MacLean, H., *Biochem. J.*, 1915, ix, 103.

⁶ Dunham, E. K., *J. Biol. Chem.*, 1908, iv, 297. Dunham, E. K., and Jacobson, C. A., *Z. physiol. Chem.*, 1910, lxiv, 302.

⁷ Levene and West, *J. Biol. Chem.*, 1917, xxxi, 635.

rial gave with orcin the typical test for galactose, and on hydrolysis yielded sphingosine and the typical fatty acid mixture.

0.0984 gm. substance gave 0.2456 gm. CO_2 and 0.0972 gm. H_2O .

0.500 " " neutralized 6 cc. 0.1 N HCl.

	Calculated for phrenosin:	Found:
C.....	69.65	69.60
H.....	11.24	11.30
N.....	1.70	1.68

The optical rotation of the substance, in a mixture of equal parts of chloroform and methyl alcohol (by volume) was:

$$[\alpha]_D^{20} = \frac{9.5312 \times 0.26^\circ}{0.3550 \times 0.5} = +14.00^\circ$$

Hydrolysis of the Mixed Cerebrosides.

1.5 gm. of the mixed cerebrosides were heated with 75 cc. of 3 per cent sulfuric acid for 24 hours in a sealed tube at 105° . The base and acids were separated and prepared for analysis as described in a previous article.

The acids had the following composition:

0.1012 gm. substance gave 0.2978 gm. CO_2 and 0.1098 gm. H_2O .

	Calculated for		Found:
	$\text{C}_{25}\text{H}_{50}\text{O}_3$:	$\text{C}_{24}\text{H}_{48}\text{O}_2$:	
C.....	75.33	78.20	75.40
H.....	12.50	13.20	12.18

Thus the acid was apparently nearly pure cerebronic acid, $\text{C}_{25}\text{H}_{50}\text{O}_3$.

The base was transformed into the sulfate and gave the following figures on analysis.

0.0990 gm. of substance gave 0.2264 gm. CO_2 and 0.0910 gm. H_2O .

	Calculated for $(\text{C}_{17}\text{H}_{35}\text{NO}_2)_2\text{H}_2\text{SO}_4$:	Found:
C.....	61.08	62.36
H.....	10.78	10.29

Crude sphingosine sulfate, previous to crystallization, very frequently gives analytical data as in the present experiment. There is little doubt that the base of the kidney cerebrosides is sphingosine.

Liver Cerebrosides.

Desiccated and pulverized liver tissue was allowed to stand over night with 95 per cent alcohol and then filtered. The residue was repeatedly extracted with boiling alcohol, each extraction lasting about $\frac{1}{2}$ hour. The combined extracts, on standing in the refrigerator, at 0° , gave a dark, nearly black deposit, which corresponds to the "white matter" of the brain extracts. This deposit was extracted in the cold progressively with acetone, alcohol, and ether. The still dark but quite dry mass was fractionated into two parts by dissolving it in hot pyridine and allowing it to cool to room temperature. The mother liquor containing the cerebrosides was concentrated and poured into acetone. The precipitate thus obtained was still very dark. For further purification it was boiled with hot alcohol; a small part remained insoluble. The solution was decanted and a concentrated solution of barium hydroxide was added as long as a precipitate formed. This mixture was allowed to stand in the ice box, and the precipitate which formed was repeatedly extracted with boiling alcohol. The extracts, upon cooling to 0° , gave a precipitate which had the appearance and properties of the brain cerebrosides. This product was then repeatedly extracted with ether, when analysis showed that it was still contaminated with large amounts of neutral fat. The purification was then continued by extraction with ether and by crystallization from acetic acid. The product, however, persisted in containing neutral fat. Finally, the product was dissolved in hot methyl ethyl ketone, from which it settled out on cooling. This was repeated three times, the product then having the composition of cerebrin. Because of the great losses connected with the purification it was not possible to obtain sufficient material for hydrolysis. The test for galactose was positive and there is little doubt that we are dealing with a characteristic cerebroside mixture.

0.1006 gm. substance gave 0.2522 gm. CO_2 and 0.1010 gm. H_2O .

	Calculated for phrenosin:	Found:
C.....	69.65	68.36
H.....	11.24	11.23

Egg Cerebrosides.

Egg yolk (dried commercial egg yolk was used in all the work) was thoroughly extracted with acetone at room temperature, to remove egg oil. The material was then extracted with boiling alcohol, repeatedly, as in the former preparations. The combined alcoholic extracts were concentrated to a small volume, and repeatedly treated with acetone, to complete the removal of the egg oil. The acetone-insoluble fraction (lecithin, cephalin, cerebrosides, and saturated phosphatides) was extracted with ether. A small part did not go into solution. The ether suspension was centrifuged, the insoluble material suspended in acetone, filtered off, and again extracted with ether. The insoluble material then corresponded to "white matter" previously mentioned, and is the material analyzed by Stern and Thierfelder⁸ and considered impure diaminomonomophosphatide. It was later given the name *albin* by Bing and Ellermann.⁹

This was fractionated out of pyridine as described above. The cerebroside fraction was crystallized out of glacial acetic acid and the neutral fat removed by repeatedly extracting with acetone at 50°, and finally by crystallization from methyl ethyl ketone. This material possessed all the physical properties of the mixed cerebrosides, and gave the galactose test with orcin.

0.1020 gm. substance gave 0.2476 gm. CO₂ and 0.0974 gm. H₂O.

0.5000 " " neutralized 6.75 cc. 0.1 N HCl.

	Calculated for phrenosin:	Found:
C.....	69.65	69.32
H.....	11.24	11.19
N.....	1.70	1.89

The optical activity of the material in a mixture of methyl alcohol and chloroform was:

$$[\alpha]_D^{20} = \frac{12.4370 \times 0.11^\circ}{0.3062 \times 0.5} = + 4.40^\circ$$

⁸ Stern, M., and Thierfelder, H., *Z. physiol. Chem.*, 1907, liii, 370.

⁹ Bing, H. J., and Ellermann, V., *Biochem. Z.* 1912, xlii, 289.

Hydrolysis of Egg Cerebrosides.

1.5 gm. of the substance were heated in a sealed tube with 75 cc. of 3 per cent sulfuric acid for 24 hours at 105°.

The acids and bases were prepared in the manner described above.

0.1000 gm. substance gave 0.2708 gm. CO₂ and 0.1070 gm. H₂O.

	Calculated for C ₂₈ H ₅₀ O ₈ :	Found:
C.....	75.33	74.52
H.....	12.50	12.09

The base was analyzed as the sulfate.

0.0905 gm. substance gave 0.2024 gm. CO₂ and 0.0810 gm. H₂O.

	Calculated for (C ₁₇ H ₃₃ NO ₂) ₂ H ₂ SO ₄ :	Found:
C.....	61.08	60.99
H.....	10.78	10.01

ON THE PRESENCE OF ALBUMOSES IN THE TISSUES AND IN THE BLOOD, WITH SPECIAL REFERENCE TO THEIR OCCURRENCE IN THE GASTRO- INTESTINAL MUCOSA.¹

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It will be admitted that it is a matter of physiological importance if it can be established (1) that albumoses are present in various organs of the body as integral components of their cells; (2) that the absorbing mucosa of the stomach and small intestine contain these substances in larger amount than other tissues, especially during the digestive period, and (3) that the plasma of the blood, the transporting agent of the body, carries these substances from the stomach and intestines to other tissues. A study of these questions which, as is well known, are not new, was forced upon us when we undertook to isolate certain active constituents of the mucosa of the stomach and the small intestine. It was not difficult to prepare from this tissue a water soluble powder which showed great physiological activity in stimulating an intestinal or uterine strip or in causing the pancreas to secrete freely. Always such powders, although entirely free from coagulable proteids, gave both the Pauly and the biuret reactions. We would note here that one of our preparations from the intestinal mucosa of the pig, which caused almost maximal contractions of the virgin guinea pig's uterus in the proportion of 1:1,000,000 and which also caused a distinct rise of blood pressure (cat), furnished a colorless aqueous solution which in these respects, as well as in its chemical reactions (biuret and Pauly reactions), behavior towards polarized light and towards saturation with ammonium sulphate, was strictly comparable to a somewhat diluted aqueous extract of the pituitary gland. This

¹ This investigation was aided by a grant from The Rockefeller Institute for Medical Research.

striking analogy led to a more careful study of the biuret-yielding substance and its identification as a secondary albumose.

That a stimulant for the intestinal musculature can be extracted from the cells of the gastric and intestinal mucosa was first shown by Enriquez and Hallion (1). It is not our purpose now to report on or analyze the great number of investigations that deal with the "peristaltic hormones" (2) that have been isolated from many organs of the body, but we wish to emphasize the fact that it is not difficult to prepare an extract of the intestinal mucosa, like that referred to above, which is highly active in the proportion of 1:1,000,000. Inasmuch as the active motiline can be present only in small amount in this extract, we may safely predict that when once it has been isolated as a chemical individual it will be found to be no less active than β -imidoazolyethylamine or than the uterine stimulant of the pituitary gland. The thought naturally arises that this powerful motor constituent of the pituitary body is possibly identical with that shown to be present in the gastric and intestinal mucosa and elsewhere in the body. The great activity of only partially purified extracts of the gastric and intestinal mucosa, which still contain a preponderance of albumoses, amino acids and inorganic salts, certainly justifies us in raising this question. We hope to be able at a later date to give something more tangible than a hypothesis on this point.²

Albumoses in the Gastric and Intestinal Mucosa.

As to the presence of albumoses in the gastric and intestinal mucosa, it is well known that Hofmeister, one of the earlier investigators in this field, maintained that "peptone" is present in these tissues. As far as is known to us, the rigid proof for such an assertion, which consists in the actual isolation of an albumose or peptone from the

² We find that Köhler (*Organextracte als Wehenmittel*, *Zentralb. f. Gynäkologie*, 1915, 891) while offering no opinion as to the identity of the substances concerned, has suggested that an extract of some organ or organs other than the hypophysis might be used instead of the latter as an oxytocic in therapeutics. In our opinion, the mucosa of the pig's stomach, from which, as already stated, a preparation that raises the blood pressure and stimulates the uterus and the intestine may readily be prepared, should furnish a substitute for the costly hypophysis preparations. Further research must determine whether this suggestion is commercially feasible.

gastric and intestinal mucosa, was not given by Hofmeister or by his pupils. Indeed, with a few exceptions (Nolf, Asher and their pupils), physiologists and bio-chemists are inclined to the opinion that the proteids of our food are absorbed only in the form of the ultimate end products of digestion—the amino acids, although the possibility that lower polypeptids may also pass into the blood is admitted.

Some have no doubt been willing to admit the taking up by the intestinal and possibly by the gastric mucosa of albumoses and peptones (the biuret-yielding polypeptids) but finding no evidence that the plasma of the portal blood is a carrier of such biuret-yielding polypeptids, these authors have fallen back on the supposition that these compounds are broken down to abiuret products, amino acids and lower polypeptids, in the mucosa itself.

Our method can be described here in outline only. Many details of the operations will be self evident to experienced workers. The treatment was the same with both gastric and the intestinal mucosa. The description here given applies specifically to the upper portion (three to four feet in length) of the small intestine of the pig, but is equally serviceable in working up the mucosa of the stomach. The intestines were brought quite fresh, many being still warm, from a slaughter house in close proximity to the laboratory. They were immediately slit open and thoroughly cleaned by agitation under a current of water until freed from adherent mucus and bile, after which the mucous membrane was scraped away with a glass plate.

The scrapings were weighed and put into large flasks and 95 per cent alcohol was introduced in small portions at a time, the flask being continually shaken, until the mixture contained from 45 to 50 per cent of alcohol. For purposes of calculation, one gram of gut scrapings was called the equivalent of one cubic centimeter of water, and in most of our work we added one liter of 95 per cent alcohol to each kilogram of gut scrapings.

The flasks, after thorough admixture of their contents, were then attached to reflux condensers, the body of the flask in each case being submerged in water, and the contents were kept at a lively boil for an hour.³ The coagulated material was now removed and pressed to a hard cake in a small wine press, and the clear or slightly turbid alcoholic filtrate, which contains our biuret substances, was concentrated to a small volume on the water bath with the assistance of an electric fan. When the alcohol and much of the water had been so far removed that the

³ As is pointed out elsewhere in this paper, a much shorter time of boiling, perhaps fifteen minutes, will suffice to dissolve out the albumoses. The above period of one hour was adhered to in experiments whose primary object was a large yield of secretine.

remaining solution had the consistency of an easily flowing syrup, it was transferred to an Erlenmeyer flask and while it was still warm and freely mobile, 95 per cent alcohol was added with a pipette in small quantities at a time with thorough agitation, so that the precipitation took place only very slowly. It is often advisable to return the flask to the water bath from time to time during the early stages of the precipitation.

When the alcoholic strength of the mixture had reached from 50 to 60 per cent at the highest the flask was set aside until the precipitate had settled out and a clear fluid could be poured off. The precipitate was then transferred to plate filters, drained at the pump, washed with 50 to 60 per cent alcohol and transferred to a large receptacle in which it could be exposed to the extracting action of 50 to 60 per cent alcohol; after a time the supernatant fluid was drawn off and worked up with the main filtrate in the manner to be described.

The alcoholic filtrates were again concentrated on the water bath under a rapidly revolving electric fan until a mobile syrup was once more obtained. This was transferred, while still hot, to large Erlenmeyer flasks and absolute alcohol was added in very small quantities at a time with thorough agitation and occasional warming until a voluminous, crumbly precipitate had settled out. At this point the alcoholic strength of the mixture had probably attained a value of 85 per cent or more, and the further addition of absolute alcohol yielded but little more of the precipitate. Nevertheless, it has been our practice to add absolute alcohol until the precipitation is finished, and in order to precipitate the biuret substance as completely as possible we added much ether at this stage. The flasks were now allowed to stand for a number of hours, or until the clear supernatant alcohol or alcohol-ether mixture could be removed with a siphon and the crumbly, though often partly sticky, precipitate could be collected on flat filters at the pump. No difficulty was encountered in disintegrating and thoroughly washing the precipitate with strong alcohol and finally with a mixture of absolute alcohol and ether. After being washed free of all fatty and other substances which are soluble in absolute alcohol and ether,⁴ the precipitate was dried *in vacuo* over sulphuric acid and ground up to a fine powder.

An aqueous solution of this powder gives the biuret reaction with great intensity. In order to remove all traces of coagulable proteid the powder was dissolved in water and a 40 per cent solution of lead acetate was added until no more precipitate fell out. To the filtrate basic lead acetate (30 per cent) was added until precipitation was complete, care being taken to avoid a large excess of this reagent. The precipitate was removed at the pump, to the filtrate 95 per cent alcohol was added until the solution attained a strength of 50 per cent and the lead was precipitated by the addition of sulphuric acid in 50 per cent alcohol. This precipitation is so complete in 50 per cent alcoholic medium that hydrogen sulphide no longer produces a blackening. After the removal of the lead sulphate by filtration and of the alcohol by evaporation, the solution was found to be free

⁴ U. S. P. ether was used for this purpose.

of native proteids. At one time we added uranyl acetate to the neutralized solution at this point in order to allay any suspicion that an alcohol-soluble proteid might have escaped the action of the above named precipitants, but the results were unaltered—the filtrate after removal of the uranium still showed the presence of proteose as before. The use of mercuric chloride as a precipitant at this point, that is, when it is added to the acidulated alcohol-water solution, also leaves our proteose in solution, again showing that it must be a substance of the character of a secondary albumose.

Further purification of the product (removal of secretine, "motiline," amino acids and primary albumoses) was effected by the use of phosphotungstic acid, mercuric chloride, silver nitrate and repeated precipitation of the final product in alcohol of varying strengths. Phosphotungstic acid (1:1) was added directly to the alcoholic, lead-free filtrate until no further precipitate settled out. This precipitation occurs in very dilute solutions, and in such solutions only the less soluble phosphotungstates, as those of the albumoses, fall out in relatively large amounts. The precipitated phosphotungstates were collected at the filter pump, washed as well as possible and pressed to a hard cake. The cake was ground up in a mortar with dilute sodium hydroxide solution until the entire cake, with the exception of an inconsiderable residue, had passed into solution. The solution should be neutral or faintly acid. In case it shows a slight turbidity a drop of alkali will at once clear it up. The phosphotungstates were now precipitated by the addition of dilute sulphuric acid, again collected in the form of a hard cake, and the process of solution in dilute alkali and reprecipitation with sulphuric acid was repeated one or more times.

The cake was then ground up in a mortar with dilute sodium hydroxide until a clear neutral or only faintly alkaline solution was obtained. Saturated barium hydroxide solution was added until a point was reached at which the hitherto suspended colloidal precipitate of barium phosphotungstate coagulated suddenly and settled quickly to the bottom of the beaker. The heavy sediment was quickly removed and the slight excess of barium was precipitated with sulphuric acid.

A 30 per cent solution of mercuric chloride in alcohol was now freely added in small portions at a time. A small quantity of a flocculent precipitate settled out on standing and was removed by filtration. To the filtrate sodium hydroxide solution was added drop by drop with constant stirring, when a whitish flocculent compound fell out and settled rapidly to the bottom of the flask. This precipitate ceases to appear when the neutral point is reached—or, more correctly, at the first indication of alkalinity—provided that an excess of mercuric chloride is present. The precipitate was collected and washed by grinding it up repeatedly in a mortar with warm water containing a little mercuric chloride until it was free from sulphates.

The mercuric precipitate was next ground up in a mortar with 0.5 per cent nitric acid. With the exception of an inconsiderable flocculent residue, the precipitate dissolved with great ease in this strength of acid. We find that this be-

havior is characteristic of the mercury compound obtained with a secondary albumose which we have prepared by digesting pig's thyroid glands with pepsin and hydrochloric acid.

The mercury may now be removed with hydrogen sulphide, although this is not always easy as the precipitate may remain in colloidal suspension. Warming on the water bath and the addition of a little nitric acid will generally induce coagulation of the mercuric sulphide. The removal of the mercury is not essential, however, as for the next step, which consists in the preparation of a silver compound, may be used either (*a*) the solution which has been freed of mercury with hydrogen sulphide or (*b*) the above mentioned 0.5 per cent nitric acid solution of the mercury compound. An excess of silver nitrate is added, and in case solution *b* has been used the precipitate of silver chloride is allowed to settle out. From the filtrate the white flocculent silver compound is precipitated out by the addition of dilute sodium hydroxide. This can be done even in the presence of mercury (solution *b*), since the silver compound falls out while the solution is still markedly acid. It is collected at the pump and thoroughly washed. Considerable loss of material may be sustained here, as water dissolves much of the silver proteose compound after the greater part of the electrolytes has been washed out of the precipitate.

The silver compound is decomposed in a mortar with a small volume of hydrochloric acid in excess and the clear filtrate is dropped into several volumes of absolute alcohol, precipitation being completed by the addition of ether. The "hydrochloride" thus thrown out is collected and washed with a mixture of absolute alcohol and ether (1:1).

In case the silver compound has been made from solution *b* (mercury still present) the final product obtained as just described will still contain a small amount of mercury which, however, is readily removable with hydrogen sulphide.

The dried hydrochloride is next dissolved in water and freed from hydrochloric acid by agitation with silver carbonate, and in this way the free albumose is obtained. At this point we may have in hand a preparation which is highly active for the virgin uterus of the guinea pig, giving an almost maximal contraction in a strength of 1:1,000,000. This is true more especially in case the phosphotungstic precipitate above described has not been subjected to reprecipitation and thorough washing. Secretine may also be present in such preparations. The ninhydrin reaction is also given promptly when a solution of the albumose is boiled for a few minutes with the reagent. The contaminating substances are removed by repeated solution in hot water and precipitation from hot absolute alcohol; also by forming a picrate, dissolving it in 20 per cent alcohol containing sulphuric acid and pre-

precipitating with absolute alcohol and ether. The free picric acid is washed away from the precipitated albumose sulphate with absolute alcohol and ether, and the albumose will now generally be found to be devoid of physiological activity. If this is not the case some of the above operations, especially the use of picric acid, should be repeated.

As thus obtained the substance has all the characteristics of a pure secondary albumose:

1. It is freely salted out when its solutions are saturated with ammonium sulphate.

2. It gives the biuret reaction, 1 mgm. to the cubic centimeter, with a fine reddish purple color. With large volumes the reaction is still good when the substance is used in the proportion of 1:10,000.

3. The Pauly reaction is immediately positive.

4. Millon's reaction is negative (with 5 mgm. to the cubic centimeter). Only thoroughly purified preparations give a negative Millon. Our notes call attention to the fact that an albumose "sulphate" which was made from a picrate may still give a positive Millon and only after being treated once more with mercuric chloride and silver nitrate will the albumose give a negative response to this reagent. We are no doubt dealing here with a number of secondary albumoses of variable composition.

5. Knop's reaction is negative (10 to 20 mgm. to the cubic centimeter). Bromine water is immediately decolorized in the performance of this reaction and a white granular bromine compound is thrown out in the presence of a little sulphuric acid.

6. Weidel's reaction is negative.

7. Addition of potassium ferrocyanide and acetic acid to a solution of the albumose causes no precipitation whatever.

8. Tannic acid gives a flocculent precipitate only when a little mineral acid is added.

9. The substance is laevo-gyrous. The specific rotation of the sulphate was found to vary from $[\alpha]_D = -72.2^\circ$ to -94.4° , according to the method of preparation.

10. The α -amino nitrogen content of a specimen of the hydrochloride, which in neutral solution failed to give the ninhydrin test (10 mgm. in the cubic centimeter), was found to be 0.53 per cent as estimated with the Van Slyke apparatus. After hydrolysis with 25 per cent

hydrochloric acid under a reflux condenser until the biuret reaction had disappeared the α -amino nitrogen was increased about five times. Unfortunately the data for the hydrolysis experiments were lost and therefore exact figures cannot be given.

11. The ninhydrin reaction is negative with the purer preparations when neutral solutions containing 10 mgm. to the cubic centimeter are boiled in the usual way with the reagent. The reaction is, however, always positive when the reagent and the albumose solution are evaporated together to dryness on the water bath and the dry residue is taken up in very weak alcohol or in water (3).

12. Solutions of the albumose may be boiled with 25 per cent hydrochloric acid or with strong nitric or sulphuric acid without developing a dark color or depositing humus substances.

Albumoses in the Gastric Mucosa.

We have found that albumoses may also readily be prepared from the well washed mucous membrane of the pig's or dog's stomach (fed animals) when the organ is worked up immediately after its removal from the body. Care was taken to keep the mucous scrapings neutral to litmus during the half hour's boiling with 50 per cent alcohol, and under these circumstances an albumose could hardly have been formed as an artefact. When the process is carried through the lead subacetate stage, with removal of the lead and precipitation with alcohol, a white powder is obtainable which contains albumoses, shows marked activity for the intestinal and uterine strip and has also some blood pressure raising power.

Relative Amounts of Albumose in the Gastric and Intestinal Mucosa of a Dog in Digestion and of a Starving Dog.

The following comparative experiments were made under as nearly identical conditions as possible on two dogs of approximately the same weight, one of which had been fed with meat six and a half hours before being killed while the other had had nothing but water for four days.

The dogs were bled to death, the stomach and small intestine (as far as the caecum) immediately taken out, slit open, cut into pieces of

convenient length and washed thoroughly, first under the tap and then in physiological salt solution, and pressed upon absorbent paper, the surface of the mucosa being gently wiped with paper.⁵

The mucous membrane of the stomach and gut was next scraped off on a glass plate and the weighed scrapings were slowly treated, with vigorous shaking, first with 50 cc. of 50 per cent alcohol and then with 95 per cent alcohol, 1 cc. of the latter being used for each gram of the scrapings.

To the resulting only slightly acid suspension was then added 6 drops of saturated sodium carbonate solution and the mixture was boiled half an hour under a reflux condenser. The supernatant liquid was poured off through a filter paper on a Büchner funnel and the coagulum boiled out three times with 125 cc. of 50 per cent alcohol, then pressed as nearly dry as possible on the funnel and finally dried to constant weight in an air bath.

The combined turbid aqueous alcoholic extracts, after removal of 10 cc. for a determination of the amount of dissolved matter, were next treated successively with neutral lead acetate, basic lead acetate and sulphuric acid, care being taken not to use too large an excess of the reagents, especially in the removal of the lead with the sulphuric acid. In each case the precipitate was first filtered off with the aid of the pump and sucked as dry as possible, and the filtrate, which was always slightly turbid, was repeatedly passed through a folded filter until clear.

The final filtrates were concentrated to a small volume on the water bath under a fan, filtered and treated, with vigorous shaking, with 10 volumes of absolute alcohol. The resulting flocculent precipitates were collected, after standing some time, as completely as possible on weighed papers, washed with 10 cc. of 10:1 alcohol, dried *in vacuo* over sulphuric acid and weighed.

The two alcohol precipitates were now dissolved in a known volume of water and sodium hydroxide and copper sulphate were added, when it was observed that the biuret color was many shades deeper in the solution derived from the fed dog than in that obtained from the starving dog. Colorimetric estimation showed that if the amount of

⁵ In the washing, the adherent bile, mucus and products of digestion were removed by rubbing the mucosa gently with the fingers.

biuret-yielding substance in the precipitate from the starving dog be taken as 1, that obtained from the fed dog must be rated as 7. Using a secondary albumose in our possession as a colorimetric standard, we found that the actual amount of albumose recovered by us from the gastro-intestinal mucosa of the starving dog was 8.2 mgm. while the amount recovered from the mucosa of the fed dog was 57.4 mgm. It is evident that whether we base our calculation on the comparative weights of the moist scrapings or on the weights of the total dry matter in them (see the table below), there is no escape from the conclusion that much more albumose is present, weight for weight, in the gastro-intestinal mucosa of the fed dog. Taking the gram of dry matter as the unit of calculation, the mucosa of the fed dog is found to contain nearly five times as much albumose as that of the starved dog.

The data from these two experiments are given in the following table.

	FED DOG	STARVED DOG
Weight of dog.....	16.5 kgm.*	15.5 kgm.
Weight of moist scrapings.....	326.5 gm.	225.5 gm.
Weight of dry matter in coagulum.....	47.8 gm.	32.7 gm.
Weight of dry matter in extract.....	13.1 gm.	8.6 gm.
Weight of total dry matter in scrapings.....	60.9 gm.	41.3 gm.
Final volume of concentrated filtrate.....	25.0 cc.	20.0 cc.
Weight of alcohol precipitate.....	415.0 mgm.	266.0 mgm.
Weight of total albumose in precipitate.....	57.4 mgm.	8.2 mgm.
Weight of albumose per gram of moist scrapings.....	0.176 mgm.	0.036 mgm.
Weight of albumose per gram of dry matter in scrapings.....	0.942 mgm.	0.199 mgm.

* Approximately. The dog was weighed after death and allowance made for the stomach, intestine and blood.

An earlier experiment with two large dogs of approximately equal weight, in which, however, no account was taken of the weight of dry matter in the coagulum and in the extracts, gave results analogous to the above although quantitatively different. In this earlier experiment the mucosa of the fed dog was found to contain about three times as much albumose as that of the starved dog.

That we do not in these experiments carry our processes through to the end and prepare the albumoses in a pure state cannot invalidate

our conclusion that the gastro-intestinal mucosa of a fed dog contains much more albumose than that of a starved dog. At the stage of our process at which the precipitates were compared, protein matter is present in the form of albumose only. Albumose which responds to the Millon reaction is still present, though later this variety may be lost in the various chemical steps that follow the use of lead subacetate.

Albumoses of the Pig's Thyroid Gland.

In order to obviate the criticism that the albumoses here described are products of autolysis we proceeded as follows in working with this gland. By special arrangement with the manager of the slaughter house we were enabled to receive the glands still warm from the hand of the butcher. The glands were cut into a few pieces and dropped at once into boiling 50 per cent alcohol. In this way approximately 1 kgm. of fresh thyroids was boiled up at the slaughter house, it being our purpose to destroy the ferments of the gland. The alcoholic solution was removed, the cut up glands were ground up in a machine and again extracted with hot 50 per cent alcohol. The alcoholic extracts were now treated in the manner described for the intestinal mucosa, up to and through the lead subacetate stage, including the removal of the lead as lead sulphate. The lead-free solution was concentrated under the electric fan at a low temperature and was then dropped into absolute alcohol and the precipitate collected and dried. This precipitate, a water-soluble white powder, gave no turbidity with potassium ferrocyanide and acetic acid, and was therefore quite free from coagulable proteid. The product was now carried through the mercuric chloride and silver nitrate treatments. It is to be noted again that the solution of the mercuric chloride precipitate in very dilute nitric acid (0.5 per cent) excludes the presence of coagulable proteid, even if the earlier steps had not already insured us against this. The silver compound was made from the nitric acid solution without first removing the mercury. From the silver compound the hydrochloride of the albumose was prepared as a dry powder in the manner already described.

This product gives the biuret reaction in the manner characteristic of albumoses, while the Millon reagent with 20 mgm. to the cubic centimeter did not give a fine red color or a red precipitate but only a

yellowish tinge. In other respects the product behaved like the albumose obtained from the intestinal mucosa. The quantity of this albumose obtainable from the thyroid gland is small as compared with that found in the intestinal mucosa. We judge that we had about 0.1 gram of dry product after having completed the lead subacetate step in our method, a quantity equivalent to 0.01 per cent of the weight of fresh bloodless glands. The amount of pure albumose present in the gland is probably lower than this figure.

Albumoses in Striated Muscle.

In order that the conditions of the extraction might be entirely under our control we made use of the skeletal muscle of the dog. The animals used had not been fed for sixteen hours. They were etherized and bled to death from the carotid artery, whereupon some of the larger muscles were rapidly stripped from the limbs and run through a meat grinder. From one animal 760 grams of ground up muscle were obtained in this way, from a second 784 grams. Each lot was worked up separately and was carried through the lead subacetate stage of our process. The lead-free solution was precipitated from an excess of absolute alcohol in the presence of a slight excess of sulphuric acid. The albumose fell out as a white flocculent precipitate, contaminated, as usual at this stage, with other constituents of the tissue, notably amino acids. It seemed hardly necessary to apply further processes of purification as it was evident that this precipitate was free of coagulable proteids. Approximately 0.01 gram dissolved in a few drops of water gave only the merest trace of precipitate with potassium ferrocyanide and acetic acid on long standing. So, too, the addition of uranyl acetate to a solution of the albumose sulphate caused the appearance of only a faint turbidity which finally aggregated to a small amount of a flocculent precipitate. The filtrate from this precipitate, after removal of the uranium with sodium hydroxide, gave the biuret reaction with great intensity.

Our experiments show that under the conditions here described only a small amount of albumose exists in dog's muscle, and it is doubtful if much more is obtainable from this tissue even when the animal is in full digestion.

Skeletal muscle tissue (920 grams) of a third dog, also unfed for

sixteen hours, was first boiled for half an hour with distilled water, then pressed to a hard cake and the filtrate discarded. The disintegrated cake was then boiled up with an equal weight of 50 per cent alcohol and the alcoholic filtrate was treated in the manner described for the intestinal mucosa up to the stage at which lead acetate is used. The powder obtained at this point (which is ordinarily the starting point for the treatment with lead acetate) failed to give a biuret reaction and it therefore was unnecessary to proceed further with this control experiment. It shows that the albumose present in the muscle was completely removed by the boiling water.

The methods heretofore applied to this tissue have failed to detect the very small amount of albumose which we have been able to extract from it with 50 per cent alcohol. Thus Whitfield (4) failed to find a proteose in rabbit's muscle, either when he treated a 0.65 per cent sodium chloride extract with trichloroacetic acid or with saturated ammonium sulphate solution. No data are given as to the quantity of tissue extract used in the separate tests. The biuret reaction is not a sensitive test for albumoses—the reaction is not obtainable with a secondary albumose in a concentration below 1:10,000 or 1:12,000. We have usually made this reaction with 0.1 per cent solutions of albumose, that is, in a concentration of 1:1,000. Then, too, a number of disturbing factors must be taken into account in applying this test. At the concentration of albumose of 1:2,000 an excess of dextrose, if present, or a yellow color in the solution will entirely cover up the biuret reaction, and a negative result under these conditions is not conclusive. It is therefore advisable to make the test with an isolated precipitate rather than with a complex solution.

v. Fürth (5) and Mays (6) also report that they could not detect a true albumose in muscle by the methods employed by them. Mays, however, was able to separate, by salting out with magnesium sulphate, considerable quantities (140 grams from 1814 grams of extract) of a proteose from a Liebig extract prepared in South America. The substance was not put through a series of purifications as in our work above, and Mays is undecided whether to call it an albumose or a gelatose. He thinks it very unlikely that it is an albumose which has been formed by the action of hot water (84° to 94°) on the chopped up meat and he falls back on the supposition that it is most likely a gela-

tose, although admitting the difficulties that stand in the way of this suggestion. In the first place, the chemical reactions cited by Mays do not speak conclusively for gelatose, and the processes employed in manufacturing the extract can lead, as Mays admits, to the conversion of gluten into gelatose only if a number of quite unproved assumptions are made in regard to the favoring actions of salts and other constituents of the extract in facilitating this conversion.

Under the circumstances and in view of our findings, we prefer to believe that the albumose of meat extracts is accounted for by the presence of this substance in the muscle tissue and that it has been concentrated in the extract by the processes of manufacture, as is the case, for example, in the pituitary extracts of commerce. Mays found that meat extract contains 7.8 per cent of his albumose. Pituitary extracts, as we have shown in an earlier paper (7), may contain fully 10 per cent of albumose.

Albumoses in the Gravid Uterus.

The uterus was removed by Caesarean section from a woman at full term by Prof. J. W. Williams and was brought to the laboratory after having lain ten hours on ice. The decidua and mucosa were scraped from the fundus and the organ was then ground up and boiled with alcohol in the usual manner. The processes were carried through the lead subacetate stage and precipitation with alcohol gave a white powder which gave a colorless solution in water and which responded to the tests for albumose already described. The yield of albumose appeared to us to be greater than in the case of skeletal muscle.

Albumoses in Other Organs.

We have not made a systematic search for albumoses in other organs. It may be of interest however, to state that an examination of a soluble extract of the *corpora lutea* which is sold to physicians by one of our large manufacturing firms behaves in respect to the proteid precipitants and reactions described in this paper in such a manner that one can only conclude that proteoses are present in the extract.

Each cubic centimeter of the extract contains the soluble constituents present in 0.2 gram of the dried corpora lutea. The solution

gives no precipitate on boiling after the addition of a trace of acetic acid, nor does it give a precipitate with potassium ferrocyanide and acetic acid; the biuret reaction is positive and gives the reddish color characteristic of albumoses; the Pauly reaction is also given immediately. From these reactions one must conclude that we are dealing here with an extract which is free from coagulable proteids but still contains a proteose in considerable quantity.

A second commercial preparation which we have not ourselves examined but which undoubtedly contains an albumose is the "Hormonal" of Zuelzer. This extract is prepared from the spleen of cattle and has been shown to have some value as a motor stimulant for the intestine. In a few instances severe collapse and even death have followed the intravenous injection of 20 cc. of the preparation. It was shown by Dittler and Mohr (8) that Hormonal contains powerful blood pressure lowering substances. The fatalities, as well as the cases of dangerous collapse, are fully accounted for by these depressor substances. We would note in passing that intestinal extracts also contain these very powerful blood pressure lowering substances and that they can be completely removed by the proper use of alcohol and basic lead acetate. Zuelzer (9) believed that the untoward effects observed were caused by the presence of a toxic albumose and took steps to have this substance removed. Dittler and Mohr (10), however, found on examining the new Hormonal that it still contains depressor substances, and the physiological chemist, Professor Siegfried, who also examined the new preparation, declared it to contain albumoses.

We have here, then, an extract of the spleen, which was no doubt prepared from perfectly fresh material, which contains albumose. That the extract still contains blood pressure lowering substances is due to faulty technique.

Albumoses in the Blood.

A number of investigators, notably Hofmeister and his school, as also E. Freund and his pupils (11), have always maintained that blood plasma and blood serum contain albumoses in small quantity. The opposite opinion has been upheld energetically by Neumeister and Abderhalden and their pupils (12). Howell also was not able to con-

vince himself that blood contains a non-coagulable proteid of a proteose nature. He found (13) that

Blood serum from the fed or starved animal when subjected to dialysis in colloidon tubes gives no indication of the presence of a perceptible amount of peptone or proteose.

That both the blood and the urine may contain albumoses in pathological states of the organism, in which absorption of broken down cells occurs on a large scale, is generally admitted, but we shall not here consider this aspect of the question.

We have repeated the work of the later investigators of the Hofmeister school, Hohlweg and Meyer, and have found, quite in agreement with their statements, that whole blood, serum or plasma which has been freed from coagulable proteids by their method gives a negative ferrocyanide and a *positive biuret test*. Apparently such fluids should contain a proteose. The biuret-yielding material can be salted out from such solutions by the use of ammonium sulphate and heat, but we have not been able to identify the proteid precipitate thus obtained as a proteose. If originally present as an albumose, it must have been changed into an insoluble dysalbumose in the course of our operations, since the final product obtained after removing ammonium sulphate from the precipitate is no longer soluble in distilled water. Such negative results were obtained even with as much as two liters of blood, both with the original method of Hohlweg and Meyer and with a modification of it devised by ourselves which obviates dilution of the plasma.

When we applied our alcohol-lead acetate-subacetate method to the plasma (oxalated or citrated), only negative results were obtained with both portal and systemic blood. One element of uncertainty inheres, however, in this negative finding by this particular method—the final product here obtained always gave a yellow solution and in such a solution it is impossible to determine with certainty, by means of the biuret reaction, whether small quantities of a proteose are present.

Positive results were obtained when this method was applied to the mass of red and white corpuscles that were obtained by centrifugalizing two liters of systemic blood. The final precipitate in alcohol,

after removal of lead by the method previously outlined, gave the tests for albumose so unmistakably that it seemed unnecessary to proceed further with the purification of the precipitate. The quantity of albumose obtainable from the cellular elements of the blood by boiling with 50 per cent alcohol is, however, not large but suffices for identification.

Dialyzates of Portal, Hepatic and Systemic Blood Plasma.

Oxalated plasma as obtained by centrifugalizing blood from the portal and the hepatic veins, as also from the carotid artery, was submitted to dialysis during periods varying from three to fifteen hours in series of celloidin sacs⁶ and tubes. The fluid on the outside of the sacs was distilled water in some experiments; in others it was an 0.8 per cent solution of sodium chloride. In the course of a few hours considerable biuret-yielding material passes through the walls of these celloidin sacs and tubes. At first precautions were taken to collect the blood in sterile fashion and to sterilize both the sacs and the vessels in which the dialysis was carried out, but as it was found that these precautions were of no advantage in experiments of short duration they were discontinued. It is possible to overlook the proteid material that passes through celloidin sacs if the biuret test is applied to the concentrated dialyzate without first removing the sugar with boiling 93 per cent alcohol. In this way dextrose and other disturbing substances are removed, and a concentrated residue which at first fails to give the biuret reaction will now give this test very beautifully.

Considerable quantities of this diffusible proteid were collected by concentrating the distilled water dialyzates of various kinds of plasma. In each case the attempt was made to isolate a proteose from the

⁶ The celloidin solution here used was like that described in the Journ. Pharm. and Exper. Therap., 1914, v, 279, also *ibid.*, p. 611. In this work on vividiffusion (1914) Abel and his associates also failed to isolate a proteose from their diffusates, in spite of the fact that a secondary albumose (Hirudin) was constantly being run into the veins of the animal used. As in the present experiments a coagulated proteid was always found in small amount in the evaporated dialyzate, which goes to show that the diffusible proteid here described exists preformed in the circulating blood.

partially concentrated fluids by the employment of lead acetate and basic lead acetate for the removal of coagulable proteids but always the attempt ended in failure. We are well aware that certain primary albumoses are precipitated by basic lead acetate and that the absence of a biuret-yielding substance in the filtrate from the lead precipitate does not exclude the presence of this class of proteoses in the dialyzates. But if they are present, they are easily changed into insoluble dysalbumoses. Skins of coagulated albumin are formed during the concentration of the dialyzates on the water bath under the electric fan, and when sufficiently concentrated exhaustion of the residue with 93 per cent alcohol causes it to become entirely insoluble in distilled water. As far as we have gone, then, *we can only say that our methods have not enabled us to separate a true proteose from the proteids that pass freely through celloidin membranes during the first few hours of dialysis.*

The inherent difficulties in the way of separating albumoses from the blood are well illustrated in the following experiment. Corrosive sublimate, as is well known, is an effective agent for removing coagulable proteids from albuminous solutions. We desired to learn if a secondary albumose added to whole blood in known quantity could be recovered when this agent is employed. Accordingly 0.15 gram of a pure secondary albumose dissolved in 7.5 cc. of 0.8 per cent sodium chloride solution was injected directly into the outflowing stream of blood from the portal vein of a dog. The portal blood with the admixed albumose was then whipped with a wire brush and filtered through cotton. The filtrate amounted to 96 cc. and should have contained the added albumose (0.15 gram) except in so far as it was absorbed by the fibrin. The defibrinated blood with its albumose was now treated according to the directions of Gayda (14). It was stirred into 480 cc. of 2 per cent mercuric chloride in 0.8 per cent hydrochloric acid, frequently shaken, let stand overnight, filtered through paper, freed from mercury with hydrogen sulphide and from the excess of the hydrogen sulphide with a current of air, neutralized with sodium hydroxide and concentrated under a fan at about 55° to a volume of 35 cc. The flocks which separated out gave no biuret reaction. The filtrate when further concentrated likewise gave no biuret reaction. So also the precipitate obtained by adding alcohol failed to give even

a trace of a biuret reaction. Evidently the added albumose was lost entirely in the course of our manipulations. It must be borne in mind that the secondary albumose here employed is not precipitated by mercuric chloride in the presence of dilute acid when tested by itself in aqueous solution.

On the other hand, when this albumose was added to oxalated plasma in the proportion of 0.005 gram to the cubic centimeter and the plasma was then dialyzed against distilled water it was not difficult to show that a little of the albumose passed into the dialyzate. Ordinarily, when the dialyzate is concentrated nearly to dryness under the electric fan at as low a temperature as possible and then exhausted with hot 93 per cent alcohol the proteids present become insoluble in water, as has already been stated. In the present instance, however, a water-soluble proteid was found in the final residue and this could only be the albumose originally added to the plasma. In conclusion, it should be stated that the amount of albumose recovered in the dialyzate appeared to be much less than we had a right to expect.

Other Instances of the Presence of Albumoses in Tissues, as Described in the Literature of Biological Chemistry.

As already mentioned in this paper, Abel and Pincoffs (7) have shown that all pituitary preparations examined by them contain albumoses.

Slowtsoff (15), in reporting the results of his analyses of human semen, writes as follows:

Human semen contains, in addition to a nucleoproteid, traces of mucin and albumin, an albumose-like substance which in respect to its precipitation limits and its chemical reactions must be regarded as a primary albumose.

Szumowski (16), who made alcoholic extracts of the finely comminuted organs of geese, doves and dogs in a search for zein which had been administered to these animals, states that he found a biuret-yielding substance which was not zein in a number of instances. He is at a loss to explain his findings. We have no doubt that had this author treated his alcoholic extracts in the manner described by us, thus removing sugar and other disturbing substances, a positive biuret reaction would have been obtained by him in many more of his extracts. We have cited this author's findings because we believe that

they are explainable only on the basis that he has unwittingly extracted albumoses from a number of tissues.

Wohlgemuth (17), who has made a study of human bone marrow under pathological conditions, precipitating the coagulable proteids by boiling with dilute acetic acid and sodium chloride up to 1 per cent until the filtrate gave no turbidity with potassium ferrocyanide, reports as follows in regard to albumoses in this tissue: Albumoses were present in one case out of five of yellow bone marrow, and out of six of red bone marrow four contained albumoses. The marrow was taken from the femur in each case.

It is not our purpose to sift the voluminous literature pertaining to the appearance of albumoses in the urine in various pathological conditions. In the great majority of instances the excreted albumoses appear to be of the secondary type (18) and there is at present good reason for believing that this is accounted for by the absorption into the blood of the constituents of disintegrating tissues. The frequent appearance of albumoses in the urine under circumstances where tissue breakdown obtains, lends support to the view that the albumoses found by us in various tissues were actually present in them and were dissolved out of them by the boiling 50 per cent alcohol used in our experiments.

Panzer (19) has isolated albumoses from the sporozoites of a species of *Coccidia* known as *Goussia gadi*.

DISCUSSION.

It has been shown that albumoses may be isolated from the tissues by extracting them with boiling 50 per cent alcohol and removing coagulable proteids from the alcoholic extract. In the case of the intestine and the thyroid gland the isolation was carried through so many processes that the secondary albumose finally obtained was as nearly a chemical individual or entity as present methods permit us to isolate, and even in the case of other tissues the isolation had proceeded far enough to dispel all doubt that we had an albumose in our hands. Where, as in the single instance of the plasma of the blood, the isolation could not be effected, we have so stated and have not accepted the positive qualitative tests of earlier investigators as proof of the existence of albumoses in this fluid.

But the actual isolation of an albumose from a tissue is in itself no proof that it was present as such in the cells of that tissue. The albumose may have been formed from the proteids of the tissue in the course of the chemical manipulations employed for their isolation. We have given special attention to this phase of the question and offer the following considerations in support of our belief that albumoses actually exist in the cells of the body, being stages in the metabolism comparable with the amino acids and other intermediary products. We employed boiling 50 per cent alcohol because it is a good solvent for our albumose and because it thoroughly disintegrates the cells of the tissues and coagulates their proteids. The time of boiling was originally set at one hour in the case of the intestinal mucosa because we desired at that time to extract completely other substances than albumose. As stated elsewhere, we have frequently extracted tissues for half an hour and we have no doubt that where it is desired to obtain only the proteoses boiling the finely divided tissue for a quarter of an hour will amply meet the requirements of the case. Weak alcohol is an indifferent fluid and can not of itself hydrolyze proteids to albumoses within the above time limits, if at all.

It may be argued that the acidity of the dead tissues was sufficient to cause the formation of small quantities of albumoses in the boiling extracts. We think that this also can not be urged as the source of the albumoses. In a number of instances, as in experiments with the gastric and intestinal mucosa of fed and starved dogs, great care was taken to boil at the neutral point to litmus and not to concentrate the alcoholic extracts until after the use of lead acetate and basic lead acetate and after the removal of lead with sulphuric acid. These precipitations were all made at room temperature and the final filtrate no longer contained a coagulable proteid at the time the alcohol and acetic acid were driven off. Even when this final alcoholic filtrate was concentrated under pressure reduced, after having neutralized the acid, the results remained the same. Finally, it is difficult to see (on the supposition that our albumose is an artefact) how so much more albumose can be obtained from the gastro-intestinal mucosa of the fed dogs than from that of starved dogs. It will be noted that the amounts of albumose which can be isolated in the two

cases stand in no relation to the amounts of proteid (total dry matter) present in the two mucosae.

Post mortem autolytic changes, while possibly responsible for a part of the albumose present in the pituitary and other extracts used in medicine, certainly play no rôle as a causative factor in the experiments described by us, in which tissues were taken directly from animals just killed. Autolysis can not therefore be held responsible for the proteoses isolated by us.

Our observation that the gastro-intestinal mucosa of fed dogs contains much more albumose than is found in the corresponding mucosa of starved dogs calls for a word of discussion. In this connection the question naturally arises whether it is possible to wash away from the mucosa all adherent proteose, retaining only that part that has entered the cells of the mucosa. We answer in the affirmative, if the washing is done under a jet of running water and the surface of the mucous membrane is stretched⁷ and gently rubbed with the finger, if the pieces of intestine are then soaked and again rubbed in large bowls containing 0.8 per cent sodium chloride solution and if, finally, the surface of the intestine is pressed down on filter paper and gently wiped with pieces of this paper. All this was done with the stomach and intestines of the dogs used in our experiments.

We also find support for our opinion that a mucous surface can be washed free from adherent proteid material in the extensive literature of physiology in which quantitative experiments are described in which loops of intestines or gastric pouches are treated as receptacles, the assumption being made that these living receptacles can be freed of their contents and washed, much as is done with laboratory utensils.

But we can offer a more incontrovertible proof of the truth of the statement that freely soluble substances, such as peptones and albumoses, can be washed away completely from the surface of the intestines, by citing our earlier experiments with certain phthaleins (20).

In these experiments 20 to 30 cc. of a 2 per cent solution of tetrachlorphthalein were injected into dogs subcutaneously. After the expiration of from eighteen to thirty hours the animals were killed, the

⁷ It is especially necessary to stretch the walls of the stomach while washing so that the folds of the mucosa may present a level surface to the impinging jet of water.

large and the small intestines were removed separately, opened and washed perfectly clean under running water. Before washing, the application of a 5 or 10 per cent solution of sodium hydroxide to the mucous membrane of the small intestine always gave an intense deep red stain over large areas. This was especially intense where mucus was abundant. On applying the alkali to the *well washed* small intestine, however, *no color reaction could be obtained*.

It only remains to add that the mucosa of the large intestine could not be washed free of the phthalein but always took on an intense, brilliant deep red stain throughout its whole extent upon the addition of alkali. Frozen sections showed that the drug was contained not only in the cells of the mucosa but was present also in the submucosa. Similar sections of the small intestine were found to be free of phthalein. Further research proved that the phthalein was excreted by the liver and was carried down the intestine in a state of solution and reabsorbed by the large intestine only, the absorption beginning abruptly at the line where the small intestine merges into the large.

When the delicacy of this color reaction is borne in mind, when we consider that the mucous surface of the small intestine, which has been bathed throughout its entire length with a solution of the phthalein, could be washed entirely free of the compound while no amount of washing could remove it from the mucosa of the large gut (the compound having entered the cells of this organ), we cannot escape the conviction that soluble and merely adherent compounds are completely removable from the mucous surfaces of the intestines or stomach.

In the light of the above experience we must conclude that that portion of proteose (as in the case of the phthalein in the large gut) which could not be washed away from the gastro-intestinal mucosa had actually been taken up by the absorbing cells.

At the present writing we can not trace the absorbed proteoses beyond the mucous membrane and, strictly speaking, it is only an inference on our part that they are absorbed as such and not produced by synthesis from absorbed amino acids. Nolf (21) and Asher (22) have shown that the intestines can take up proteoses, and we know that these derivatives of proteids are always present in the digestive canal during digestion. It seems far fetched, therefore, to assume that the

increased amount of proteose found by us in the gastro-intestinal mucosa of fed dogs was not absorbed as such but was made by synthesis. Certainly, the assumption that these soluble proteoses are taken up by the cells from the digested matter with which they are in contact seems the more rational point of view.

There is much controversy in this field. Abderhalden (23), in a review of the subject in the last edition of his text book, after weighing the facts pro and con, leaves it undecided whether higher peptids are absorbed by the intestinal mucosa and does not commit himself unreservedly to the theory that amino acids only are absorbed. E. Zunz (24) shows that proteoses disappear both from the stomach and the intestines but concludes that one can not decide how much of the absorbed nitrogen is taken up in the form of higher split products, how much in the form of lower, because of the reversible action of ferments, and so leaves the matter in an unsettled state. London (25) and his collaborators maintain that no absorption takes place from the stomach during digestion, while Tobler (26), Lang (27), Salaskin (28), Scheunert (29) and Grimmer (30) all hold that the products of the gastric digestion of proteids (proteoses) are absorbed in the stomach, merely differing among themselves as to the quantitative factor in the absorption.

The amount of proteoses or higher polypeptids taken up by the gastric and intestinal mucosa during a digestive period must be very considerable, as judged by our findings. In the case of the amino acids it has been shown that the mesenteric blood may contain twice as much amino acids after feeding with meat as before feeding (31). But in the case of our albumoses we are halted at the point of intake and cannot follow them in their passage into the plasma. As we have seen, we were not able to isolate a proteose from plasma. For the present, therefore, we can only speculate as to the fate of the proteoses that have entered the cells of the gastro-intestinal mucosa. Are our methods for separating them from the proteids of portal plasma at fault? Are they split up into abiuret products in the mucosa and do they pass into the blood stream only in the form of such abiuret products? Are they built up in the mucosa into *less diffusible proteids which have lost most of their proteose characteristics?* The knowledge at hand permits of no conclusive answer to these

questions. Nevertheless, it must be regarded as a step forward that so marked an increase in the proteose content of the gastro-intestinal mucosa can be shown to take place during digestion. This observation (if it be granted that these proteoses are actually absorbed and not produced by synthesis) certainly militates against the theory now so widely advocated that proteids are broken down completely into amino acids in the digestive canal and absorbed only in this form.

SUMMARY.

1. Albumoses can be isolated in varying amounts from the tissues of the body, inclusive of the cellular elements of the blood. The methods employed did not, however, enable us to separate a proteose of any kind from the plasma of the blood.

2. To prepare an albumose from the gastric or intestinal mucosa which is entirely devoid of pharmacological activity (pressor, oxytotic and secretory) requires the employment of numerous chemical procedures, as outlined in this paper.

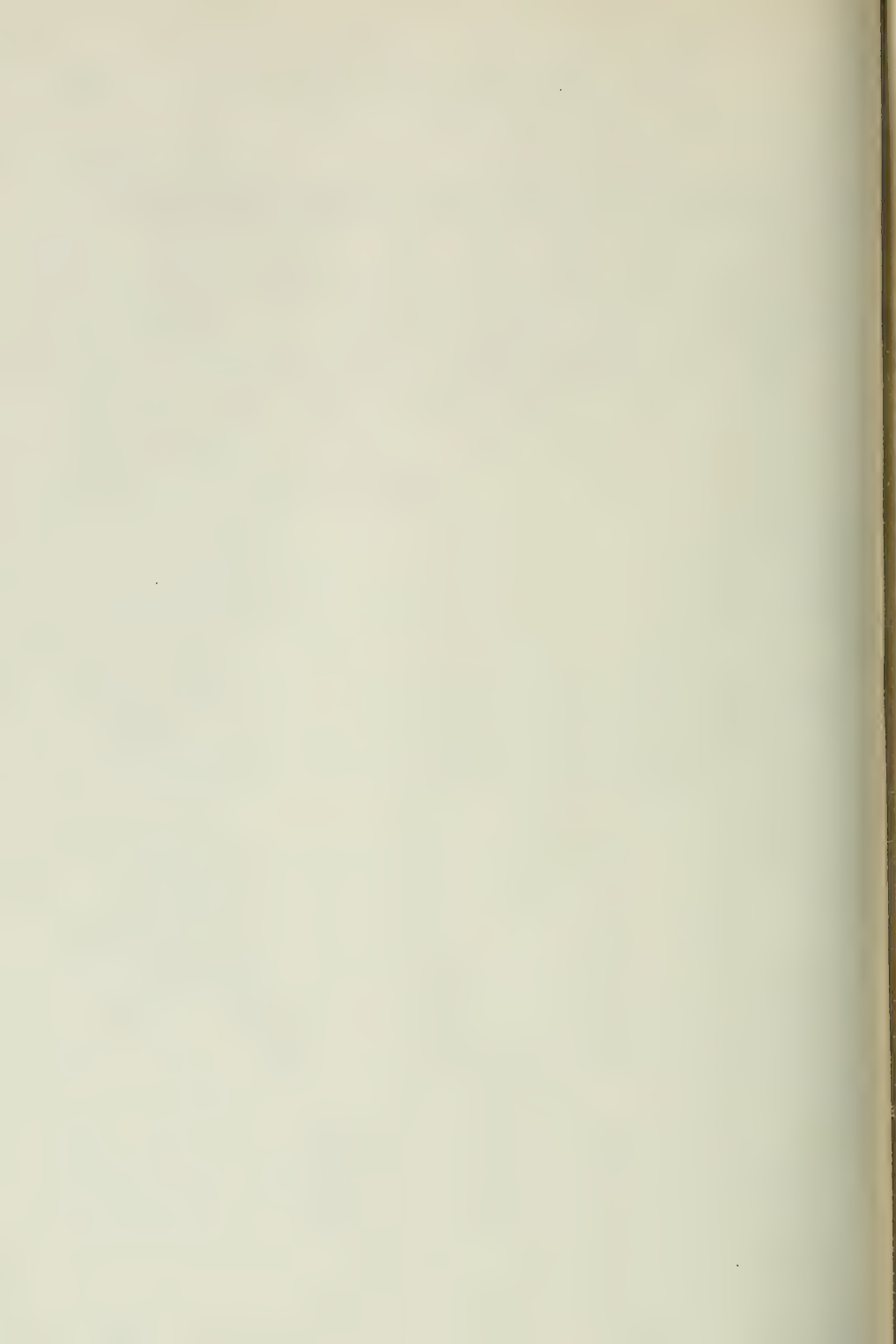
3. The gastro-intestinal mucosa can be washed free of soluble adherent substances, such as the proteoses. We are justified in assuming that what can not be washed away must be truly a part of the mucosa. The gastro-intestinal mucosa contains from three to five times as much albumose during digestion of meat as after deprivation of all food (except water) for four days.

4. In view of this finding we can not accept the theory that proteids are taken up by the absorbing surfaces of the digestive apparatus only in the form of amino acids, but must believe rather that proteoses as well as amino acids are freely absorbed, as has long been maintained for the former by certain investigators. While we are able to trace the further passage of amino acids from the mucosa to various organs via the blood current, we find it impossible at present to do this with proteoses. These can not be traced further than into the absorbing mucosa, unless it should be assumed that the cellular elements of the blood are distributing agents for them—a point of view which we are at the moment not justified in advancing. The details of the fate of the absorbed proteoses still remain to be determined.

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ON NITRO- AND AMINOPHENOXYACETIC ACIDS.

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In the course of synthetic work which was undertaken in connection with investigations to be published later, it was found necessary to prepare a large number of substituted aminophenoxyacetic acids and their derivatives. A perusal of the literature showed that only a few of the simpler members of this series had been described, so that the preparation and study of these substances became a separate undertaking. We wish, therefore, to present the results of this work in the present communication.

At first we adopted the procedure of starting with the appropriate nitrophenol, converting this into the nitrophenoxyacetic acid, and then into the desired amino derivative. In preparing the nitrophenoxyacetic acids it was found most satisfactory to employ the method used by Kym¹ for the preparation of *p*-nitrophenoxyacetic acid. This involves heating the dried sodium salt of the nitrophenol with ethyl chloroacetate and saponifying the nitro ester so obtained. Although this method is somewhat more time-consuming than the reaction between the nitrophenol and chloroacetic acid in boiling alkaline solution it proved to be more economical in the case of the less easily accessible nitrophenols, since the yields of the esters were practically quantitative.

Later in the work an attempt was made to prepare the nitrophenoxyacetic acid from 4-nitroguaiacol, but no reaction took place between the dried sodium salt and ethyl chloroacetate. It was found, however, that the method used by Howard² for the preparation of *p*-aminophenoxyacetic acid was applicable in this case. In this method the acetaminophenol and chloroacetic acid are allowed to re-

¹ Kym, *J. prakt. Chem.*, [2] 55, 113 (1897).

² Howard, *Ber.* 30, 546 (1897).

act in boiling aqueous solution with two molecular equivalents of sodium hydroxide. This method was also used for the preparation of a number of the aminophenoxyacetic acids which had already been prepared over the nitro compounds, and in these cases also not only gave excellent results but seemed even better suited for large-scale work than that involving the use of the nitrophenol sodium salts. The method was accordingly adopted with equal success in all subsequent work in which accessible aminophenols were in question. In the preparation of acetaminophenoxyacetic acids by this method we have found it advantageous, after the first reaction between the acetaminophenol, chloroacetic acid, and alkali, to add again one-half the amounts of chloroacetic acid, alkali and water, and again boil to neutral reaction. The yields then obtained were usually excellent.

The acetaminophenols necessary in this investigation were easily prepared from the aminophenols according to the method of Lumière and Barbier,¹ in which the aminophenol and acetic anhydride are made to react in aqueous solution. For the preparation of such *p*-aminophenols as were used as starting materials we have adopted with excellent results the rapid and convenient method used by Henderson and Sutherland² for the preparation of *p*-aminothymol, consisting in the reduction of the nitrosophenol in ammoniacal solution with hydrogen sulfide. In all cases in which a phenol can be easily converted into its nitroso derivative (or quinoneoxime) we regard this method as the most convenient for quickly preparing the corresponding *p*-aminophenol in large amounts.

At the end of the paper we have appended a number of closely related substances, such as naphthoxy- and quinolyloxyacetic acids and two aminophenoxybutyric acids. These last were prepared by converting the bromopropyl ethers of the acetaminophenols into the nitriles and then saponifying. Although the reaction between the bromopropyl ethers and potassium cyanide proceeded normally, our attempts to prepare the corresponding amino- β -phenoxypropionic acids failed, owing to the fact that the acetaminophenyl bromethyl ethers react with potassium cyanide in a different sense, recalling a

¹ Lumière and Barbier, *Bull. soc. chim.*, [3] 33, 783, (1905).

² Henderson and Sutherland, *J. Chem. Soc.*, 97, 1617 (1910).

similar failure to obtain nitrile formation reported by Schreiber¹ in attempting to react potassium cyanide with *p*-cresoxyethyl bromide. The substitution of β -iodo- or bromopropionic acid or ester for chloroacetic acid or ester in the methods given above also proved unsatisfactory.

In general, the aminophenoxyacetic acids described in this paper are difficultly soluble in cold water, more or less readily in hot, and sparingly soluble in the neutral organic solvents. Aqueous suspensions dissolve on adding either mineral acid, alkali, carbonate, or ammonia. Practically all of the aminophenoxyacetic acids and their esters give colors with ferric chloride, and those containing primary amino groups are readily diazotizable, coupling with R-salt to give soluble dyes of varying shades of red. The amino acids also form hydrochlorides which crystallize readily but are very easily hydrolyzed. Finally, several of the acids showed the property of retaining one molecule of water, even at 100° *in vacuo*.

As in our previous papers, all substances were dried to constant weight *in vacuo* over a suitable drying agent, at 100° whenever possible, before analyses or melting-point determinations were made.

(A) *o*-Aminophenoxyacetic Derivatives.

o-Acetaminophenoxyacetic Acid, $o\text{-CH}_3\text{CONHC}_6\text{H}_4\text{OCH}_2\text{CO}_2\text{H}$.—*o*-Aminophenol was prepared from the nitrophenol by means of sodium sulfide and converted into the acetyl derivative by dissolving in one mol of dilute sodium hydroxide, adding 1.1 mols acetic anhydride, and then acidifying, according to Lumière and Barbier.² 40 g. *o*-acetaminophenol, 25 g. chloroacetic acid (1 mol), 42.6 g. 50% sodium hydroxide solution (2 mols), and about 200 cc. water were boiled down to small volume in an open flask. One-half the above quantities of chloroacetic acid, alkali, and water were then added and the solution once more boiled down to small volume. After diluting with water, hydrochloric acid was added in excess, the acetamino acid separating as a thick slurry. This was filtered off, washed with water and dried in the air. The yield was 50 g. Recrystallized

¹ Schreiber, *Ber.*, 24, 196 (1891).

² *Loc. cit.*

from water it forms large, faintly pinkish spears which contain one molecule of water of crystallization and which melt with gas evolution when plunged into a bath at a temperature above 100° . When anhydrous and recrystallized again from boiling xylene, in which it is very difficultly soluble, it melts at $153-4^{\circ}$ (cor.) with preliminary softening. The compound is very easily soluble in cold acetic acid when not anhydrous, and sparingly soluble after its water of crystallization is driven off.

1.1467 g. subst.: 0.0904 g. loss *in vacuo*, finally at 100° . H_2O , 7.88%. $1\text{H}_2\text{O}$, 7.93%.

Anhydrous: Kjeldahl: 0.3190 g. subst.; 15.30 cc. 0.1 *N* HCl.

Calc. for $\text{C}_{10}\text{H}_{11}\text{O}_4\text{N}$: N, 6.70%. Found: N, 6.72%.

o-Aminophenoxyacetic Anhydride.—This substance is obtained by boiling the acetamino acid with 1:1 hydrochloric acid, the anhydride separating after a few minutes. The anhydride may also be prepared from *o*-nitrophenoxyacetic acid by the usual methods of reduction, including that involving ferrous sulfate and ammonia. We have found it advantageous, however, to substitute sodium hydroxide for the ammonia in the case of this particular substance, owing to the sparing solubility of the anhydride in boiling, dilute ammonia and its ready solubility, on the other hand, in boiling dilute sodium hydroxide. By adopting this modification, yields as high as 78% of the theory could be obtained, or about 10% in excess of the amounts recovered when ammonia was used. The rapidity and convenience of the procedure here outlined would make this appear to be the most satisfactory method for the preparation of the anhydride, especially as the necessary *o*-nitrophenoxyacetic acid may be easily obtained as follows: 139 g. *o*-nitrophenol are suspended in 800 cc. water, 160 g. 50% sodium hydroxide solution (2 mols) added, and the mixture warmed until the phenol dissolves. A solution of 95 g. (1 mol) of chloroacetic acid in 200 cc. water is then added and the whole boiled under a reflux condenser for 1.5 hours. The unchanged nitrophenol is then distilled off with steam and the residual solution cooled, filtered, and acidified to Congo red with hydrochloric acid. A number of preparations, carried out as above, gave yields of from 55 to 60 g. of *o*-nitrophenoxyacetic acid.

o-Aminophenoxyacetic anhydride was first satisfactorily isolated

by Thate,¹ who reduced the nitro acid with iron filings and acetic acid and reported the melting point as 166–7°. Our samples of the anhydride showed all the properties described by Thate, except the melting point, which was found to be 173–3.5° (cor.) after recrystallization from water.

(B) *m*-Aminophenoxyacetic Acids.

(1) *m*-Aminophenoxyacetic Acid.

m-Nitrophenoxyacetic Acid, $m\text{-O}_2\text{NC}_6\text{H}_4\text{OCH}_2\text{CO}_2\text{H}$.—35.9 g. of the anhydrous sodium salt of *m*-nitrophenol and 27.4 g. chloroacetic ethyl ester were heated in an oil bath under an air condenser, first at 130–140° until a clear melt was obtained, and finally for 2 hours at 175°. After cooling, the melt was taken up in alcohol and the ester saponified by adding an excess of aqueous sodium hydroxide and heating the mixture on the water bath for 15 minutes. On acidifying with hydrochloric acid, the *m*-nitrophenoxyacetic acid separated in a yield of 46 g. Recrystallized from water, the acid forms brilliant, slightly brownish needles which melt constantly at 154–5° (cor.) with preliminary softening, resolidifying when the temperature of the bath drops a few degrees below the melting point. The acid dissolves readily in water only at the boiling point, is easily soluble in acetic acid and difficultly in toluene, benzene or chloroform. Bischoff,² who obtained the compound by the hydrolysis of the corresponding malonic ester, gives 152–3° as the melting point, while Hewitt, Johnson, and Pope,³ who obtained the ester in 40% yield by heating *m*-nitrophenol and chloroacetic ester in alcohol in the presence of sodium, give 151° as the point of fusion.

0.1982 g. subst.; 12.4 cc. moist N, 766 mm., 22°.

Calc. for $\text{C}_8\text{H}_7\text{O}_6\text{N}$: N, 7.11%. Found: N, 7.11%.

m-Aminophenoxyacetic Acid.—41.4 g. *m*-nitrophenoxyacetic acid were dissolved in warm alcohol and treated first with 112 cc. concentrated hydrochloric acid and then with 37.6 g. tin, added in small por-

¹ Thate, *J. prakt. Chem.*, [2] 29, 178 (1884).

² Bischoff, *Ber.*, 40, 3143 (1907).

³ Hewitt, Johnson and Pope, *J. Chem. Soc.*, 103, 1626 (1913).

tions to the warm solution. When a drop of the mixture gave a clear solution on dilution with water, the liquid was decanted off, evaporated to dryness, taken up in water, and the tin precipitated with hydrogen sulfide. The filtrate from the tin sulfide was evaporated to dryness, yielding 37.5 g. of the crude amino acid hydrochloride. A portion of this was dissolved in water, boiled with bone-black, filtered, cooled, and the free amino acid precipitated by the addition of sodium acetate solution. Recrystallized first from water, then by dissolving in dilute hydrochloric acid and precipitating with sodium acetate, it forms colorless, lenticular platelets which dissolve only sparingly in the usual neutral solvents. Rapidly heated to 205°, then slowly, it melts at 207–8° with decomposition. The aqueous suspension of the amino acid gives a dull brown color with ferric chloride on warming.

0.1574 g. subst.; 11.85 cc. moist N, 748 mm., 24°.

Calc. for $C_8H_9O_3N$: N, 8.38%. Found: N, 8.27%.

m-Aminophenoxyacetic Ethyl Ester Hydrochloride.—The crude amino acid hydrochloride obtained above was suspended in about 10 parts of absolute alcohol and treated with a stream of dry hydrochloric acid gas, without cooling. The mixture was concentrated *in vacuo* and the salt, which had separated partially, was completely precipitated with dry ether. A portion was recrystallized from a small volume of absolute alcohol, using bone-black, and letting the filtered solution stand at 0°. The salt separates as colorless plates and flat needles, which melt at 135–6.5° with slight preliminary softening and evolve gas above 200°. It is very readily soluble in water, the free oily ester separating from the solution on adding sodium carbonate.

0.1353 g. subst.; 0.0837 g. AgCl.

Calc. for $C_{10}H_{13}O_3N.HCl$: Cl, 15.31%. Found: Cl, 15.31%.

(2) *z-Methyl-5-Aminophenoxyacetic Acid*.

2-Methyl-5-nitrophenoxyacetic Acid.—The dry sodium salt of 2-methyl-5-nitrophenol was prepared from equimolecular amounts of the nitrocresol and alcoholic sodium hydroxide by evaporating to dryness *in vacuo*, then in a desiccator, and finally in an air bath at 140°. 55.2 g. of the salt thus obtained were heated in an oil bath under an

air condenser with 38.7 g. chloroacetic ester (1 mol) until clear at 130–40° and finally for 2 hours at 175°. The melt was taken up in alcohol, diluted with an equal volume of water, and the ester saponified by warming with an excess of sodium hydroxide. The nitro acid, precipitated by means of sulfuric acid, weighed 49.8 g. Recrystallized first from acetic acid, then water, it forms delicate, drab-colored needles which melt at 177–7.5° (cor.) with preliminary softening. It dissolves sparingly in cold water, acetic acid, or benzene, and readily in alcohol or acetone.

0.1617 g. subst.; 9.6 cc. moist N, 756 mm., 21.5°.

Calc. for $C_9H_9O_5N$: N, 6.64%. Found: N, 6.67%.

2-Methyl-5-aminophenoxyacetic Acid.—This substance is mentioned in Ger. pat. 230,592,¹ but we have been unable to find a description of its preparation or properties. 2-Methyl-5-nitrophenoxyacetic acid was reduced with tin and hydrochloric acid and the amino acid hydrochloride isolated exactly as in the case of *m*-aminophenoxyacetic acid. The salt was taken up with water, filtered, and the free acid precipitated by addition of sodium acetate solution. Recrystallized by dissolving in dilute hydrochloric acid and precipitating with sodium acetate, it forms sheaves of minute spindles which decompose at about 232° with preliminary softening. It is difficultly soluble in the usual neutral solvents and gives no color with ferric chloride.

0.1444 g. subst.; 9.8 cc. N, 761 mm., 21.0°.

Calc. for $C_9H_{11}O_3N$: N, 7.74%. Found: N, 7.70%.

(3) *4-Methyl-5-aminophenoxyacetic Acid*.

4-Methyl-5-nitrophenoxyacetic Acid.—The sodium salt of 4-methyl-5-nitrophenol was prepared by dissolving the nitrocresol in the theoretical amount of 2 N sodium hydroxide solution and salting out with sodium chloride. The phenoxyacetic acid was prepared from the dried salt exactly as in the two preceding cases. The yield was practically theoretical. Recrystallized first from water, using bone-black, then from acetic acid, the compound melts at 151–4° (cor.) with preliminary softening. It separates from toluene in feathery

¹ Friedländer, *Fortschr. Teerfarbenfabrik.*, **10**, 880.

aggregates of very faintly yellow crystals which melt as above. The acid dissolves in acetic acid or hot toluene, giving colorless solutions, while the solution in boiling water is yellow.

0.1680 g. subst.; 10.1 cc. N, 760 mm., 21.0°.

Calc. for $C_9H_9O_5N$: N, 6.64%. Found: N, 6.81%.

4-Methyl-5-aminophenoxyacetic Acid.—The nitro acid was reduced as in previous examples. The amino acid was isolated from the crude hydrochloride by taking this up in water, filtering, and adding sodium acetate. Recrystallized from water, it forms long, narrow, slightly brownish leaflets which soften above 200° and melt with decomposition at 235–40°. It is very difficultly soluble in the usual neutral solvents. A solution in hot water gives, with ferric chloride, a purplish color, changing through brown to green and depositing dark green flocks.

0.1403 g. subst.; 9.3 cc. N, 763 mm., 25.5°.

Calc. for $C_9H_{11}O_3N$: N, 7.74%. Found: N, 7.61%.

(4) *3-Amino-6-methoxyphenoxyacetic Acid*.

3-Nitro-6-methoxyphenoxyacetic Ethyl Ester.—The sodium salt of 5-nitroguaiacol was prepared from 5-nitroguaiacol and alcoholic sodium hydroxide. Recrystallized from 85% alcohol, it forms orange-red, glistening needles which are easily soluble in water and burn explosively when placed in a flame. 28.7 g. of the dried salt were heated for about 1.5 hours in an oil bath at 150° with 18.4 g. chloroacetic ester (until the melt was clear) and then at 180° for about 2 hours. The melt was cooled, taken up with benzene, filtered from sodium chloride, and precipitated with ligroin. The yield of ester was 33 g. Recrystallized twice from 95% alcohol and twice from benzene it forms faintly yellow rhombs which melt at 84.5–5° (cor.) with slight preliminary softening. The ester is sparingly soluble in the cold in 95% alcohol or dry ether, and dissolves in benzene or toluene.

Kjeldahl: 0.2562 g. subst.; 9.9 cc. 0.1 N HCl.

Calc. for $C_{11}H_{13}O_6N$: N, 5.49%. Found: N 5.41%.

3-Nitro-6-methoxyphenoxyacetic Acid.—The ester was dissolved in 50% alcohol and warmed on the water bath with an excess of sodium

hydroxide for one-half hour. The solution was cooled, diluted with water, and the nitro acid precipitated with sulfuric acid. After filtering, washing with water, and drying, the yield was 37 g. from 35.5 g. of the dry sodium salt of 5-nitroguaiacol. Recrystallized successively from 85% alcohol, acetic acid, and 95% alcohol, the nitro acid forms glistening, cream-colored, microscopic prisms which sublime slightly above 150° and melt at 184.5–6° (cor.) with preliminary softening. It is very difficultly soluble in the cold in water, alcohol, chloroform, benzene, or toluene.

Kjeldahl: 0.1899 g. subst.; 8.15 cc. 0.1 N HCl.

Calc. for $C_9H_9O_6N$: N, 6.17%. Found: N, 6.01%.

3-Amino-6-methoxyphenoxyacetic Acid.—10 g. of the nitro acid were dissolved in dilute aqueous ammonia, warmed, and added to a hot solution of 90 g. of crystallized ferrous sulfate in 230 cc. water. Ammonia was added in excess and the mixture heated for about 15 minutes before filtering. The filtrate was acidified with acetic acid and concentrated to small bulk. The amino acid separated in a yield of 6 g. Purified by dissolving in a little dilute hydrochloric acid, boiling with bone-black, filtering, and adding sodium acetate to the filtrate, then by recrystallization from water, the substance forms clusters of minute needles which contain between 0.5 and one molecule of water of crystallization. It is very difficultly soluble in the usual neutral solvents and, when anhydrous, melts with preliminary decomposition at 222–4°. In aqueous suspension the amino acid gives a deep violet color with ferric chloride, while it dissolves in concentrated sulfuric acid with an indigo color.

Kjeldahl: 0.1995 g. subst.; 9.4 cc. 0.1 N HCl.

Calc. for $C_9H_{11}O_4N$: N, 6.51%. Found: N, 6.60%.

The amino acid was also prepared through the acetamino derivative by the following series of reactions:

5-Acetaminoguaiacol (3-Acetamino-6-methoxyphenol).—The hydrochloride of 5-aminoguaiacol was prepared by reduction of the nitro compound according to Mameli.¹ The acetamino derivative was obtained by dissolving the hydrochloride in water, adding an excess of

¹ Mameli, *Chem. Zentr.*, 1908, I, 25.

sodium acetate, and then shaking with 1.1 mols of acetic anhydride, essentially as described by Jona and Pozzi.¹ The product obtained by us, however, differed in its properties from those recorded by these authors. Recrystallized twice from 95% alcohol it forms glistening, rhombic prisms, which melt at 169–72° with preliminary softening, instead of 116–9°. It is difficultly soluble in cold water, or hot benzene, but dissolves more readily in the cold in alcohol or acetone. An aqueous suspension gives a slowly developing olive-brown color with ferric chloride and dissolves with a pink color, changing to brown, when a drop of sodium hydroxide solution is added.

0.1333 g. subst.; 9.0 cc. N, 763 mm. 22.5°.

Calc. for $C_9H_{11}O_3N$: N, 7.74%. Found: N, 7.83%.

3-Acetamino-6-methoxyphenoxyacetic Acid.—84 g. 5-acetaminoguaiacol and 44 g. chloroacetic acid were dissolved in 800 cc. water containing 37.2 g. sodium hydroxide (2 mols) and slowly boiled down to small volume in an open flask. The solution was cooled, acidified with acetic acid, filtered from any unchanged acetaminoguaiacol, and then acidified to Congo red with mineral acid. An excellent yield of the phenoxyacetic acid was obtained. Formed in this way, the acid separates as a hydrate which melts at about 110° and is easily soluble in acetic acid, but on rubbing the solution in this solvent or recrystallizing even from water an anhydrous form, crystallizing in rhombs, is obtained. A portion, recrystallized first from water, then from acetic acid, melted at 208–10° (cor.) with preliminary softening. The anhydrous acid is difficultly soluble in the cold in the usual neutral solvents. When boiled with 1:1 hydrochloric acid, it yields the above amino acid.

Kjeldahl: 0.2950 g. subst.; 12.10 cc. 0.1 N HCl.

Calc. for $C_{11}H_{13}O_5N$: N, 5.86%. Found: N, 5.75%.

(C) *p*-Nitro- and *p*-Aminophenoxyacetic Acids.

Especially in the case of the *p*-aminophenoxyacetic acids is it preferable, as stated in the introduction, to proceed over the corresponding acetamino compounds, owing to the ease with which the

¹ Jona and Pozzi, *Gaz. chim. ital.*, 41, I, 729 (1911).

parent aminophenols may be prepared. Of the methods available, we have found it very satisfactory, both from the standpoint of yield and of convenience, to nitrosate phenols containing a free *para* position and then reduce the nitrosophenols in ammoniacal solution with hydrogen sulfide, according to the method used by Henderson and Sutherland¹ in the case of *p*-nitrosothymol.

(1) *p*-Aminophenoxyacetic Acid.

p-Aminophenoxyacetic Acid.—The *p*-aminophenol necessary for the preparation of this substance was prepared as above indicated and converted into the acetyl derivative by the method of Lumière and Barbier.¹ 177 g. *p*-acetaminophenol, 111 g. chloroacetic acid, 188 g. 50% sodium hydroxide solution, and 1700 cc. water were boiled down to somewhat less than one-half volume in an open flask. The solution was then treated with one-half the original amounts of chloroacetic acid, sodium hydroxide, and water, and again boiled down to small volume. The acetaminophenoxyacetic acid was precipitated by acidifying to Congo red and was washed with water. The yield was 205 g., corresponding in properties to those given by Howard,¹ who omitted the second treatment with chloroacetic acid and alkali, a procedure which, however, we regard as essential for attaining high yields. The crude hydrochloride of *p*-aminophenoxyacetic acid was obtained by boiling the acetamino compound with 1:1 hydrochloric acid. The amino acid itself melts with gas evolution and subsequent resolidification at about 220°.²

The Methyl Ester Hydrochloride.—217 g. of crude *p*-aminophenoxyacetic acid hydrochloride were treated in 2 liters of dry methyl alcohol with a rapid stream of dry hydrochloric acid gas, without cooling. The ester hydrochloride separated and was filtered off and washed with dry ether. Additional fractions were obtained on concentrating the alcoholic filtrate *in vacuo*, the total yield being 205 g. A portion was recrystallized from absolute alcohol, forming long, broad needles which melted with effervescence at 223–5° when rapidly heated. The salt dissolves readily in water or methyl alcohol and only sparingly in cold absolute alcohol.

¹ *Loc. cit.*

² Cf. THIS JOURNAL, 39, 1437 (1917).

0.2803 g. subst.; 0.1852 g. AgCl.

Calc. for $C_9H_{11}O_3N \cdot HCl$: Cl, 16.30%. Found: Cl, 16.34%.

The Methyl Ester.—A portion of the crude ester hydrochloride was dissolved in a little water and decomposed with sodium carbonate solution. The ester separated as an oil which rapidly crystallized. Recrystallized from water, it forms long needles which soften above 63° and melt at $65-6^\circ$ (cor.). It is difficultly soluble in cold water and readily in cold alcohol or benzene. The aqueous solution gives a purple color with ferric chloride.

Kjeldahl: 0.3226 g. subst.; 17.7 cc. 0.1 *N* HCl.

Calc. for $C_9H_{11}O_3N$: N, 7.74%. Found: N, 7.69%.

(2) *p*-Methylaminophenoxyacetic Acid.

p-Acetylmethylaminophenoxyacetic Acid, $p\text{-CH}_3\text{CON}(\text{CH}_3)\text{C}_6\text{H}_4\text{-OCH}_2\text{CO}_2\text{H}$.—*p*-Acetylmethylaminophenol was prepared by dissolving "metol" in water, adding 1.1 mols of acetic anhydride, turbinizing, and adding an excess of sodium acetate. Equimolecular amounts of the phenol and chloroacetic acid and 2 mols of sodium hydroxide were boiled in 10 parts of water under a reflux condenser until neutral, about 2 hours being required. The solution was cooled, filtered from a small amount of unchanged phenol, and acidified to Congo red with hydrochloric acid. The phenoxyacetic acid, obtained in excellent yield, was filtered off and washed with water. Recrystallized twice from water, it forms almost colorless prisms which melt at $151-2^\circ$ (cor.) with slight preliminary softening. The acid dissolves in ethyl acetate or absolute alcohol, but is only sparingly soluble in the cold in water or acetic acid, and practically insoluble in toluene or chloroform.

Kjeldahl: 0.3300 g. subst.; 14.55 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{13}O_4M$: N, 6.28%. Found: N, 6.17%.

p-Methylaminophenoxyacetic Acid.—The acetyl compound was boiled several hours with 5 parts 1:1 hydrochloric acid, concentrated *in vacuo* to a syrup, taken up with water, and treated with an excess of sodium acetate solution. The amino acid separated at once and was washed with water and alcohol and dried. In one series of ex-

periments 70 g. *p*-methylaminophenol sulfate ("metol"), carried through the steps described above, yielded 60 g. of the amino acid. Recrystallized twice from water, it forms almost colorless, glistening scales which soften above 200° and melt at 213–4° with gas evolution. The substance is very difficultly soluble in the usual neutral solvents. An aqueous suspension gives a deep lilac color with ferric chloride, while a solution in dilute hydrochloric acid deposits glistening needles of a nitroso compound when treated with sodium nitrite solution.

Kjeldahl: 0.3108 g. subst.; 17.0 cc. 0.1 *N* HCl.

Calc. for $C_9H_{11}O_3N$: N, 7.74%. Found: N, 7.66%.

(3) *p*-Glycinephenoxyacetic Acid.

p-Glycine Ethyl Ester Phenoxyacetic Acid, $p\text{-H}_5\text{C}_2\text{O}_2\text{CCH}_2\text{NHC}_6\text{H}_4\text{-OCH}_2\text{CO}_2\text{H}$.—12 g. of air-dry *p*-aminophenoxyacetic acid were dissolved in 67 cc. of normal sodium hydroxide and boiled under a reflux condenser with 8.2 g. chloroacetic ethyl ester and 70 cc. alcohol for 1.5 hours. After cooling the ester separated on rubbing and was filtered off and washed with a little alcohol. Recrystallized twice from 85% alcohol, using bone-black, it forms almost colorless, radiating masses of hair-like needles which melt and decompose at 173–6° with preliminary softening. The ester gives a strong iodoform test and dissolves readily in water, the solution giving a violet color with ferric chloride.

Kjeldahl: 0.2117 g. subst.; 8.8 cc. 0.1 *N* HCl.

Calc. for $C_{12}H_{13}O_5N$: N, 5.53%. Found: N, 5.82%.

p-Glycinephenoxyacetic Acid.—The ethyl ester was warmed on the water bath for 10 minutes with a slight excess of double normal sodium hydroxide solution, cooled, acidified to Congo red with hydrochloric acid and concentrated *in vacuo*. The addition of strong hydrochloric acid caused a precipitate of the hydrochloride of the amino acid to separate on scratching. This was filtered off, dissolved in a small volume of hot water, and treated with saturated sodium acetate solution. The precipitate was filtered off and recrystallized from water, using bone-black, forming crusts consisting of spherules of micro-crystals. When rapidly heated to 175° and then slowly, the acid melts at 177–80° with gas evolution, a mixture with the ester melting at about 160°. When pure, the acid is rather difficultly solu-

ble in cold water, acetic acid, or 95% alcohol. The aqueous solution gives a deep violet color with ferric chloride.

Kjeldahl: 0.2294 g. subst.; 14.3 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{11}O_6N$: N, 6.22%. Found: N, 6.23%.

The glycinephenoxyacetic acid was also prepared directly from *p*-aminophenoxyacetic acid, chloroacetic acid, and alkali, but, as made by this method, it could not be obtained analytically pure by recrystallization from water or 50% alcohol, probably owing to the presence of a more difficultly soluble diglycinephenoxyacetic acid. For synthetic purposes, however, the acid was sufficiently pure, as was shown by its conversion into the dimethyl ester.

The Dimethyl Ester.—10.6 g. of the crude acid were treated in 100 cc. of dry methyl alcohol with a stream of dry hydrochloric acid gas. The ester hydrochloride separated on cooling, the process being completed by the addition of dry ether. The yield was 10 g. A portion of the salt was decomposed with sodium carbonate solution, the gummy precipitate solidifying on rubbing. Recrystallized twice from methyl alcohol, it forms almost colorless, transparent prisms which melt at $63.5-4^\circ$ (cor.) with slight preliminary softening. The ester is rather sparingly soluble in the cold in alcohol, methyl alcohol, or ether, but dissolves readily in benzene or acetone. The aqueous suspension gives a slowly developing violet color with ferric chloride, while a solution in acid yields an orange-colored precipitate with sodium nitrite.

0.1819 g. subst.; 8.8 cc. N, 766 mm., 26.5° .

Calc. for $C_{12}H_{15}O_6N$: N, 5.53%. Found: N, 5.37%.

(4) 2-Methyl-4-aminophenoxyacetic Acid.

2-Methyl-4-acetaminophenoxyacetic Acid.—*o*-Cresol was nitrosated in aqueous solution by means of sulfuric acid and sodium nitrite and the nitroso compound reduced in 10% ammoniacal solution with hydrogen sulfide. The resulting *p*-amino-*o*-cresol was acetylated as in previous examples. 62.5 g. *p*-acetamino-*o*-cresol, 40 g. chloroacetic acid, and 33.7 g. sodium hydroxide were dissolved in 500 cc. water, boiled down in an open flask to a volume of about 200 cc., and then boiled under a reflux condenser until neutral. The solution was cooled

and acidified with hydrochloric acid. The yield of acetamino acid was 75% of the theory. Recrystallized twice from acetic acid, it melts with preliminary softening at 202–4.5°. It is very difficultly soluble in the cold in the usual solvents.

Kjeldahl: 0.3071 g. subst.; 13.05 cc. 0.1 N HCl
Calc. for $C_{11}H_{13}O_4N$: N, 6.28%. Found: N, 5.95%.

2-Methyl-4-nitrophenoxyacetic Acid.—Equimolecular amounts of the dried sodium salt of *p*-nitro-*o*-cresol and ethyl chloroacetate were heated as in previous examples and the crude ester saponified in the usual way. Recrystallized twice from 50% acetic acid, then from toluene, the nitro acid forms practically colorless, felted needles which melt with preliminary softening at 127.5–30.5° to a liquid which clears completely at 135.5°. It is easily soluble in alcohol, hot 50% acetic acid, or hot toluene.

0.1369 g. subst.; 8.15 cc. moist N, 774 mm., 21.7°.
Calc. for $C_9H_9O_5N$: N, 6.64%. Found: N, 6.85%.

2-Methyl-4-aminophenoxyacetic Acid.—This substance was obtained both by reduction of the nitro acid in aqueous-alcoholic hydrochloric acid by means of tin, and by saponification of the acetyl derivative with 1:1 hydrochloric acid. The acid was separated from the solution of its hydrochloride by means of sodium acetate and purified by taking up in dilute hydrochloric acid and again adding sodium acetate solution. The amino acid forms almost colorless needles which sinter at about 230° and then darken, but do not melt up to 285°. It is difficultly soluble in the usual neutral solvents, and, in aqueous suspension, gives a deep violet color with ferric chloride. The substance apparently separates with one molecule of water of crystallization which is not lost at 100° *in vacuo*.

0.1254 g. subst.; 7.6 cc. N, 760 mm., 19.5°.
Calc. for $C_9H_{11}O_3N \cdot H_2O$: N, 7.04%. Found: N, 7.08%.

The Methyl Ester Hydrochloride.—The salt was prepared in the same way as in previous cases, completing the precipitation by the addition of dry ether. Recrystallized from absolute alcohol, it forms minute needles which melt and decompose at 195–200° with prelim-

inary softening. The salt is readily soluble in water, the solution giving a gradually deepening purple color with ferric chloride.

0.2748 g. subst.; 0.1679 g. AgCl.

Calc. for $C_{10}H_{13}O_3N.HCl$: Cl, 15.31%. Found: Cl, 15.12%.

The Methyl Ester.—Sodium carbonate separates the ester from the salt as an oil which solidifies on scratching. Dried and recrystallized from ligroin, the only one of the usual organic solvents in which it is difficultly soluble, it forms colorless needles which melt at $59.5-60^\circ$ (cor.) with slight preliminary softening.

Kjeldahl: 0.3077 g. subst.; 0.1525 cc. 0.1 N HCl.

Calc. for $C_{10}H_{13}O_3N$: N, 7.18%. Found: N, 6.92%.

The Ethyl Ester Hydrochloride.—This was prepared in the same way as the methyl esters, concentrating the solution to small bulk *in vacuo* and precipitating the salt with dry ether. Recrystallized by dissolving in absolute alcohol and precipitating with dry ether, it forms colorless, glistening needles and plates, which melt at about $185-93^\circ$ with gas evolution and preliminary softening.

0.1540 g. subst.; 11.63 cc. $AgNO_3$ soln.¹

Calc. for $C_{11}H_{15}O_3N.HCl$: Cl, 14.44%. Found: Cl, 14.01%.

The Ethyl Ester.—Sodium carbonate separates the ester from an aqueous solution of the salt as an oil which soon solidifies. Recrystallized first from ligroin, then by dissolving in a little hot benzene, adding ligroin cautiously, and cooling, the ester separates as pale brown prisms which melt at $43-4.5^\circ$ (cor.) with preliminary softening. It is less easily soluble in ligroin than in the other organic solvents.

0.0793 g. subst.; 4.9 cc. N, 756 mm., 26.5° .

Calc. for $C_{11}H_{15}O_3N$: N, 6.70%. Found: N, 6.77%.

(5) 3-Methyl-4-aminophenoxyacetic Acid.

3-Methyl-4-acetaminophenoxyacetic Acid.—*p*-Acetamino-*m*-cresol was prepared through the nitroso compound of *m*-cresol by reduction with ammonia and hydrogen sulfide and acetylation of the amino-

¹ 1 cc. = 0.00186 g. Cl.

phenol as in the preceding examples. 21 g. acetamino compound, 13.3 g. chloroacetic acid, and 11.2 g. sodium hydroxide were dissolved in 300 cc. water and boiled down to about 100 cc., continuing the boiling under a reflux condenser until neutral. The solution was then cooled and the acetamino acid precipitated by adding hydrochloric acid. The yield was 21 g. Recrystallized from water, it forms aggregates of flat, glistening prisms, which, after a subsequent recrystallization from acetic acid, melt at $165-7.5^{\circ}$ with preliminary softening. The acid is difficultly soluble in the cold in water or acetic acid.

0.3174 g. subst.; 13.70 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{13}O_4N$: N, 6.28%. Found: N, 6.05%.

3-Methyl-4-nitrophenoxyacetic Acid.—This substance was prepared in the usual way from the dried sodium salt of *p*-nitro-*m*-cresol and a slight excess of chloroacetic ester, followed by saponification. The yield was 87% of the theory. Recrystallized first from 50% alcohol, with bone-blackening, then from acetic acid, the nitro acid forms faintly yellow needles which become opaque on drying and melt at $141-3^{\circ}$.

0.1124 g. subst.; 6.6 cc. N, 764 mm., 26.0° .

Calc. for $C_9H_9O_5N$: N, 6.64%. Found: N, 6.52%.

3-Methyl-4-aminophenoxyacetic Acid.—This substance was prepared as in the case of its isomer, both by reduction of the nitro acid with tin and aqueous alcoholic hydrochloric acid and, more conveniently, by hydrolysis of the acetamino acid.

Recrystallized from water it forms slightly brownish, hexagonal plates which contain one molecule of water of crystallization. The acid decomposes partially below the melting point, which lies at about $217-9^{\circ}$. In aqueous solution it gives a violet color with ferric chloride. The acid is very difficultly soluble in benzene or cold water.

1.2986 g. subst.; 0.1192 g. loss, 100° in *vacuo* H_2O , 9.18%. 1 H_2O , 9.05%.

Anhydrous: Kjeldahl: 0.2296 g. subst.; 13.0 cc. 0.1 *N* HCl.

Calc. for $C_9H_{11}O_3N$: N, 7.74%. Found: N, 7.93%.

3-Methyl-4-aminophenoxyacetic Methyl Ester Hydrochloride.—On esterification of the acid in dry methyl alcohol saturated with hydrochloric acid the salt separated as large, glistening plates, more being obtained by addition of dry ether. Recrystallized by dissolving in

dry methyl alcohol and precipitating with dry ether, it melts and effervesces at 195–200° with slight preliminary softening.

0.2410 g. subst.; 0.1504 g. AgCl.

Calc. for $C_{10}H_{13}O_3N.HCl$: Cl, 15.31%. Found: Cl, 15.43%.

The Methyl Ester.—The crude ester, precipitated from the salt by means of sodium carbonate, was recrystallized from 85% alcohol, forming rosets of glistening needles which soften above 104° and melt at 105–5.5° (cor.). The base is readily soluble in methyl alcohol, less easily in ether.

Kjeldahl: 0.2874 g. subst.; 14.25 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{13}O_3N$: N, 7.18%. Found: N, 6.95%.

The Ethyl Ester Hydrochloride.—This was prepared in the same way as its 2-methyl isomer. Recrystallized twice from absolute alcohol, in which it is rather difficultly soluble, it forms rhombic crystals, which melt at about 203–4° with gas evolution and preliminary softening.

0.2102 g. subst.; 0.1227 g. AgCl.

Calc. for $C_{11}H_{15}O_3N.HCl$: Cl, 14.44%. Found: Cl, 14.43%.

The Ethyl Ester.—Sodium carbonate deposited the ester from an aqueous solution of the salt as an oil which crystallized after several days in the ice box. The product was dried, taken up in alcohol-free ether, and the solution filtered and allowed to evaporate spontaneously in the ice box. The crystalline portion of the residue was freed from oil by pressing out on a porous plate and was recrystallized twice from alcohol-free ether with the aid of a freezing mixture. The ester forms pale brown needles which soften above 53.5° and melt at 55–5.5° (cor.). It dissolves readily at room temperature in the usual organic solvents except ligroin.

Kjeldahl: 0.2765 g. subst.: 12.85 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{15}O_3N$: N, 6.70%. Found: N, 6.51%.

3-Methyl-4-chloroacetylaminophenoxyacetic Acid.—6.25 g. of the amino acid were suspended in water and dissolved by adding a solution of sodium hydroxide, drop by drop. An excess of sodium acetate was next added, followed by 5 cc. chloroacetyl chloride, with shaking

and chilling. A thick paste of the sodium salt of the chloroacetyl compound resulted. This was dissolved by adding water and the solution neutralized with sodium hydroxide and again shaken with 3 cc. chloroacetyl chloride. The mixture was then made acid to Congo red, the chloroacetyl derivative separating as a voluminous mass of hair-like needles. After recrystallization from water the yield was 5.5 g. Recrystallized first from acetic acid, then from toluene, the substance forms rosetts of delicate needles which melt at $159-60.5^{\circ}$ (cor.) with slight preliminary softening. It is difficultly soluble in the cold in water, acetic acid, or toluene. When recrystallized from acetic acid it contains solvent of crystallization which is only slowly lost in the air, three weeks being required in one instance.

Kjeldahl: 0.2972 g. subst.; 11.50 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{12}O_4NCl$: N, 5.44%. Found: N, 5.42%.

(6) *2-Allyl-4-aminophenoxyacetic Acid*.

o-Allylphenol, *o*- $H_2C:CHCH_2C_6H_4OH$.—This substance was encountered by Claisen¹ on decomposing 3-allylsalicylic acid by means of heat, but was not described in detail. The substance is easily obtained in good yield by the general method discovered by Claisen² for the conversion of the allylethers of phenols into their corresponding *o*-allylphenols. Owing to the comparatively low boiling point of phenyl allyl ether the isomerization takes somewhat longer than in most of the cases given by Claisen,³ but is nevertheless practically quantitative. When the temperature of the boiling liquid no longer rises ($219-20^{\circ}$ is the maximum we observed) the product is dissolved in alkali, shaken out with ligroin if necessary, and the aqueous solution acidified with sulfuric acid. The allylphenol is taken up in ligroin, washed with water, filtered, dried over sodium sulfate, and evaporated. On fractionating *in vacuo* practically the entire amount boiled over at $109-10^{\circ}$ at 22 mm. Claisen gives $96-100^{\circ}$ at 12 mm. The phenol solidifies in a freezing mixture to a mass of crystals which melt at -6° . An aqueous suspension instantly reduces permanga-

¹ Claisen, *Ann.*, **401**, 73 (1913).

² *Ibid.*, p. 21.

³ *Ibid.*, p. 49.

nate, and, with ferric chloride, gives a transitory dull blue color, changing to a muddy, greenish brown.

0.1031, 0.1062 g. subst.; 0.3040, 0.3138 g. CO_2 ; 0.0701, 0.0734 g. H_2O .

Calc. for $\text{C}_9\text{H}_{10}\text{O}$: C, 80.55%; H, 7.52%. Found: C, 80.42, 80.59%; H, 7.61, 7.74%.

p-Nitroso-*o*-Allylphenol.—50 g. *o*-allylphenol were dissolved in 5 liters of water containing 375 cc. of normal sodium hydroxide solution and 170 g. sodium nitrite added. After adding ice, the mixture was vigorously turbined while adding, drop by drop, a solution of 125 cc. acetic acid in 1250 cc. water, adding ice from time to time so as to keep the temperature below 5° . The nitroso compound separates at first as a dark tar which finally crystallizes. After stirring until the entire product is crystalline, the mixture is allowed to stand in the cold for about 2 hours. The supernatant liquid is then decanted off and the precipitate filtered, washed, and purified by taking up in 20% sodium carbonate solution, filtering from tar, and acidifying with sulfuric acid. As obtained in this way, the yield of nitroso compound was 35 g. Recrystallized from toluene, it forms yellow-brown crystalline aggregates which soften above 97.5° and melt with partial decomposition at 99.5 – 100° . The substance is very difficultly soluble in cold toluene, very easily in hot, and readily soluble in cold absolute alcohol. It dissolves in sodium hydroxide, sodium carbonate, or ammonia solutions with a brown-orange color, and in sulfuric acid-phenol (Liebermann test) with an olive green color.

0.1518 g. subst.; 11.4 cc. N, 761 mm., 24.0° .

Calc. for $\text{C}_9\text{H}_9\text{O}_2\text{N}$: N, 8.59%. Found: N, 8.39%.

2-Allyl-4-aminophenol.—34 g. *p*-nitroso-*o*-allylphenol were dissolved in 350 cc. dilute ammonia (1 part concentrated to 1.5 parts water) and saturated with hydrogen sulfide. The aminophenol was filtered off, washed with water, taken up in dilute hydrochloric acid, filtered from sulfur, and reprecipitated by means of ammonia. The yield was 25 g. Recrystallized from 50% alcohol, it forms delicate, slightly brownish leaflets which soften above 111° and melt at 112.5 – 13.5° (cor.). It dissolves readily in absolute alcohol or acetone and difficultly in cold benzene, readily on warming. An aqueous suspension gives a slowly developing purple color, followed by a brown precipi-

tate, with ferric chloride. A solution in dilute alkali changes through green and violet to brown.

Kjeldahl: 0.1972 g. subst.; 13.35 cc. 0.1 *N* HCl.

Calc. for $C_9H_{11}ON$: N, 9.40%. Found: N, 9.48%.

2-Allyl-4-acetaminophenol.—25 cc. acetic anhydride were added to a solution of 25 g. of the aminophenol in 200 cc. of normal hydrochloric acid, followed, with vigorous stirring, by an excess of saturated sodium acetate solution. The acetamino compound separated as an oil which soon crystallized. The yield was 26 g. Recrystallized from a large volume of boiling benzene, it forms lenticular platelets which soften at 92° and melt at 93–3.5° (cor.). It is very easily soluble in acetic acid, alcohol, or ether. A solution in the first reduces permanganate instantly, while a suspension in water gives a momentary blue color with ferric chloride, changing to greenish gray.

Kjeldahl: 0.2999 g. subst.; 15.50 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{13}O_2N$: N, 7.33%. Found: N, 7.24%.

2-Allyl-4-acetaminophenoxyacetic Acid.—24 g. of the crude acetaminoallylphenol, 11.3 g. chloroacetic acid, 19.2 g. 50% sodium hydroxide solution, and 200 cc. water were boiled down to small bulk in an open flask. One-half of the above quantities of chloroacetic acid, alkali, and water were then added and the solution again boiled down to small volume. After dilution with warm water the solution was made just acid with acetic acid, bone-blackened, filtered, and made acid to Congo red. The acetamino acid separated as an oil which rapidly crystallized. The yield was 30 g. A small portion was recrystallized twice from acetic acid and then from xylene, forming almost colorless, wedge-shaped crystals which melted at 181–3° (cor.). The acid dissolves in acetone or acetic acid in the cold and is only very sparingly soluble in water or xylene at their boiling points. A solution in acetic acid reduces permanganate instantly, showing the allyl group to be still intact.

Kjeldahl: 0.2940 g. subst.; 11.70 cc. 0.1 *N* HCl.

Calc. for $C_{13}H_{15}O_4N$: N, 5.62%. Found: N, 5.58%.

2-Allyl-4-aminophenoxyacetic Acid.—10 g. of the crude acetamino acid were boiled for 20 minutes under an air condenser with 50 cc. 25%

sulfuric acid, a clear solution being eventually obtained. This was cooled and treated with 20% sodium carbonate solution until most of the free acid was neutralized and a small amount of tar separated. Sodium acetate solution was then added in excess, the amino acid separating and rapidly crystallizing. The crude product was suspended in water, dissolved in a slight excess of hydrochloric acid, bone-blackened, and filtered. The amino acid separated from this solution on adding an excess of sodium acetate as spherules of microscopic leaflets which dissolved with difficulty in the usual neutral solvents. The yield was 4.7 g. When rapidly heated to 190°, then slowly, the acid softens and finally melts with gas evolution at 193.5–4° to a red liquid. An aqueous suspension gives a slowly-developing, deep violet color with ferric chloride. The diazonium solution couples with R-salt to yield a much deeper red than is obtained in the case of the other aminoalkylphenoxyacetic acids.

Kjeldahl: 0.1965 g. subst.; 9.5 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{13}O_3N$: N, 6.76%. Found: N, 6.77%.

(7) *2, 5-Dimethyl-4-aminophenoxyacetic Acid.*

p-Acetamino-*p*-xylenol (2,5-dimethyl-4-acetaminophenol).—The necessary *p*-amino-*p*-xylenol was obtained in practically quantitative yield by the reduction of *p*-nitroso-*p*-xylenol in ammoniacal solution by means of hydrogen sulfide. The amino compound was converted into its acetyl derivative by dissolving in 1 mol hydrochloric acid, adding 1.25 mols acetic anhydride, turbinizing, and adding an excess of sodium acetate. The yield was almost quantitative. Recrystallized successively from water, acetic acid, and absolute alcohol, it forms transparent octahedra which soften at 178.5° and melt at 180–1.5° (cor.). The substance is practically insoluble in benzene, sparingly soluble in cold water, and more readily soluble in the cold in acetone, acetic acid, or alcohol. An aqueous solution gives a dull blue color with ferric chloride.

Kjeldahl: 0.3165 g. subst.; 17.50 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{13}O_2N$: N, 7.82%. Found: N, 7.75%.

2,5-Dimethyl-4-acetaminophenoxyacetic Acid.—This was prepared as in previous examples, adding additional amounts of chloroacetic acid,

alkali, and water after the first heating. 37 g. were obtained from 33.6 g. of the acetaminoxylan. Recrystallized first from water, then from acetic acid, the acetamino acid forms rosetts of needles which melt, with preliminary softening, at $195-7^{\circ}$ (cor.) to a yellow liquid. It is somewhat soluble in cold acetone, very sparingly in benzene or water.

Kjeldahl: 0.3119 g. subst.; 12.85 cc. 0.1 N HCl.

Calc. for $C_{12}H_{13}O_4N$: N, 5.91%. Found: N, 5.77%.

2,5-Dimethyl-4-aminophenoxyacetic Acid.—This substance was formed in quantitative yield on boiling the acetamino compound with 5 parts of 1:1 hydrochloric acid for 2 hours. The hydrochloride, which separated on cooling, yielded the free amino acid when dissolved in water and treated with sodium acetate. Recrystallized from water, the amino acid forms almost colorless, glistening scales which melt and decompose at $210-5^{\circ}$ when rapidly heated, resolidifying and then not melting below 280° . It is very sparingly soluble in the usual neutral solvents, and, in aqueous suspension, gives an ultramarine color with ferric chloride.

Kjeldahl: 0.3798 g. subst.; 19.20 cc. 0.1 N HCl.

Calc. for $C_{10}H_{13}O_3N$: N, 7.18%. Found: N, 7.08%.

The Methyl Ester Hydrochloride.—The salt separated practically quantitatively on saturation of a methyl alcoholic suspension of the acid with hydrochloric acid gas. Recrystallized from dry methyl alcohol, it forms prismatic needles which darken and soften above 220° and melt with decomposition at $232-4^{\circ}$. It is quite soluble in water, difficultly in cold absolute alcohol.

0.2534 g. subst.; 0.1478 g. AgCl.

Calc. for $C_{11}H_{13}O_3N.HCl$: Cl, 14.44%. Found: Cl, 14.43%.

The Methyl Ester.—The ester separates as an oil which crystallizes on rubbing. Recrystallized twice from methyl alcohol it forms long, flat needles, which melt at $66.5-7^{\circ}$ (cor.) with slight preliminary softening. It is readily soluble at room temperature in the usual organic solvents except ligroin, and only sparingly soluble in methyl alcohol at 0° .

Kjeldahl: 0.3050 g. subst.; 14.50 cc. 0.1 N HCl.

Calc. for $C_{11}H_{13}O_2N$: N, 6.70%. Found: N, 6.66%.

2,5-Dimethyl-4-aminophenoxyacetic Ethyl Ester Hydrochloride.—The salt separated from the ethyl alcoholic-hydrochloric acid reaction mixture on standing. Recrystallized from absolute alcohol, it forms long, glistening needles which, when rapidly heated, melt at 205–15° with decomposition and preliminary softening.

0.3125 g. subst.; 0.1729 g. AgCl.

Calc. for $C_{12}H_{17}O_3N.HCl$: Cl, 13.66%. Found: Cl, 13.69%.

The Ethyl Ester.—The base separates as an oil which solidifies on rubbing. Recrystallized first from 50% alcohol, then twice from ligroin, it forms long needles which melt at 66–6.5° (cor.) with preliminary sintering, practically the same temperature as the melting point of the methyl ester. A mixture of the two substances melted at about 50°. The ethyl ester is readily soluble in the usual organic solvents at room temperature, with the exception of ligroin. Aqueous suspensions of both esters give violet colors with ferric chloride.

Kjeldahl: 0.3096 g. subst.; 13.15 cc. 0.1 N HCl.

Calc. for $C_{12}H_{17}O_3N$: N, 6.28%. Found: N, 5.95%.

(8) *2-Methyl-4-amino-5-isopropylphenoxyacetic Acid.*

Acetaminocarvacrol.—32 g. aminocarvacrol were suspended in 200 cc. water, 12 cc. acetic acid added, and the mixture then turbined and treated with 25 cc. acetic anhydride. The acetyl derivative separated as a thick oil which gradually crystallized and then showed the properties recorded in the literature. The yield was 38.5 g.

2-Methyl-4-acetamino-5-isopropylphenoxyacetic Acid.—This substance was obtained in practically quantitative yield by the method given in previous cases. The acid separated as an oil which rapidly crystallized. Recrystallized from much water it forms glistening needles with a faint pink tinge which are difficultly soluble in the cold in neutral solvents. The acid melts constantly at 190–1.5° (cor.) with preliminary softening.

Kjeldahl: 0.3899 g. subst.; 14.50 cc. 0.1 N HCl.

Calc. for $C_{14}H_{19}O_4N$: N, 5.28%. Found: N, 5.21%.

2-Methyl-4-amino-5-isopropylphenoxyacetic Acid.—This was obtained in quantitative yield by boiling the acetamino acid with 5 parts of 1:1

hydrochloric acid for 2 hours, cooling, filtering off the hydrochloride, and recovering the remainder from the filtrate by concentration. The acid was liberated from the salt as in previous examples and was purified by a repetition of the process. The amino acid forms faintly purplish prisms which soften above 215° and melt at $225-6^{\circ}$ with effervescence. It is very difficultly soluble in the usual neutral solvents and, in aqueous suspension, gives an ultramarine color with ferric chloride.

Kjeldahl: 0.3178 g. subst.; 14.25 cc. 0.1 *N* HCl.

Calc. for $C_{12}H_{17}O_3N$: N, 6.28%. Found: N, 6.28%.

The Methyl Ester Hydrochloride.—This was prepared as in previous cases. A portion of the salt was dissolved in boiling absolute alcohol, cooled, and precipitated with dry ether, forming flat rods which softened above 180° and melted at $185-6^{\circ}$ (cor.). It is rather sparingly soluble in the cold in absolute alcohol or water, and in solution in the latter, gives a slowly-developing blue-violet color with ferric chloride.

0.3295 g. subst.; 0.1712 g. AgCl.

Calc. for $C_{13}H_{19}O_3N \cdot HCl$: Cl, 12.96%. Found: Cl, 12.85%.

The Methyl Ester.—The base separated as an oil which crystallized on standing overnight in the ice box. Recrystallized from ligroin, it formed silky, hair-like needles which melted at $29-30^{\circ}$ (cor.) and dissolved readily in the other organic solvents. The ester is strongly triboelectric.

0.0739 g. subst.; 4.0 cc. N, 763 mm., 24.5° .

Calc. for $C_{13}H_{19}O_3N$: N, 5.91%. Found: N, 6.05%.

(9) *3-Methyl-4-amino-6-isopropylphenoxyacetic Acid.*

p-Acetaminothymol.—*p*-Aminothymol was prepared according to Henderson and Sutherland¹ and acetylated as described above in the case of its isomer, acetaminocarvacol.

3-Methyl-4-acetamino-6-isopropylphenoxyacetic Acid.—The acid was obtained in the usual way, the yield being somewhat less than in the case of the isomeric, 2,4,5-compound. Recrystallized from 50% acetic acid, it forms glistening needles which melt at $186.5-88^{\circ}$ (cor.)

¹ *Loc. cit.*

with slight preliminary softening. It is difficultly soluble in water or toluene, easily in acetic acid or acetone.

Kjeldahl: 0.3457 g. subst.; 12.80 cc. 0.1 *N* HCl.

Calc. for $C_{14}H_{19}O_4N$: N, 5.28%. Found: N, 5.19%.

3-Methyl-4-amino-6-isopropylphenoxyacetic Acid.—The amino acid was prepared as in the case of the isomer. Recrystallized from ethyl acetate, it forms slightly brownish aggregates of rhombs which melted and decomposed at 204–6° when rapidly heated. It is very difficultly soluble in the usual neutral solvents and, in aqueous suspension, gives an ultramarine color with ferric chloride, as in the case of its isomer.

Kjeldahl: 0.3181 g. subst.; 14.10 cc. 0.1 *N* HCl.

Calc. for $C_{12}H_{17}O_3N$: N, 6.28%. Found: N, 6.21%.

The Methyl Ester Hydrochloride.—The salt was isolated in almost quantitative yield by evaporating to dryness *in vacuo* the methyl alcoholic-hydrochloric acid solution obtained in the esterification of the acid, taking up in a little hot absolute alcohol, and precipitating with dry ether. The salt separated slowly in sheaves and rosets of delicate needles which dissolved readily in methyl or ethyl alcohols and which, after purifying by a repetition of the final steps above, melted at 169–71° with slight preliminary softening. The aqueous solution, like the acid, gives an ultramarine color with ferric chloride. On addition of sodium carbonate the ester separated as an oil which did not crystallize.

0.2825 g. subst.; 0.1464 g. AgCl.

Calc. for $C_{13}H_{19}O_3N.HCl$: Cl, 12.96%. Found: Cl, 12.82%.

(10) *2-Bromo-4-Aminophenoxyacetic Acid*.

2-Bromo-4-nitrophenol.—Van Erp¹ brominated *p*-nitrophenol in acetic acid solution, but obtained a pure product only after precipitating the accompanying dibromo compound by means of aniline. As will be seen below, removal of this substance can be readily accomplished simply by adding water. 97.3 g. *p*-nitrophenol were dissolved

¹ Van Erp, *Rec. trav. chim.*, **29**, 187 (1910).

in about 250 cc. acetic acid and treated in several portions with a solution of 35 cc. bromine in 35 cc. acetic acid. The solution was then warmed on the water bath for several hours, until a test portion, when diluted with water, gave an oily precipitate which crystallized on cooling and scratching. An equal volume of water was next added, the solution cooled and allowed to stand for about 2 hours. The precipitated dibromo compound was filtered off and the filtrate diluted with a further quantity of water, precipitating the 2-bromo-4-nitrophenol. This was dried (yield, 83 g.) and recrystallized from toluene, then melting at $113-4^{\circ}$, as given for the product obtained by Diels and Bunzl¹ by hydrolysis of the ethyl ether.

*2-Bromo-4-aminophenol Hydrochloride.*²—This substance was obtained by adding the nitrophenol in small portions to a solution of stannous chloride in concentrated hydrochloric acid. The double tin salt separated at once and was filtered off and decomposed in the usual manner. The yield of the aminophenol hydrochloride was excellent. When rapidly heated it darkens above 230° and decomposes to a purple tar at about $260-5^{\circ}$, corresponding in its other properties to those given by Hölz.²

2-Bromo-4-acetaminophenol.—This was prepared from the hydrochloride in aqueous solution with acetic anhydride and sodium acetate as in numerous previous examples, and showed the properties given by Hölz.

2-Bromo-4-acetaminophenoxyacetic Acid.—46.3 g. of the bromoacetaminophenol, 19 g. chloroacetic acid, 16.1 g. sodium hydroxide, and 400 cc. water, were boiled down to small bulk in an open flask, diluted with water, and made just acid with acetic acid. After filtering from traces of unchanged bromoacetaminophenol the phenoxyacetic acid was precipitated by acidification to Congo red. Recrystallized twice from acetic acid the compound forms slightly brownish aggregates of minute prisms which soften above 200° and melt to a brown liquid at $216-9.5^{\circ}$ (cor.). It is somewhat less difficultly soluble at room temperature in absolute alcohol than in the other usual solvents.

¹ Diels and Bunzl, *Ber.* **38**, 1491 (1905).

² Hölz, *J. prakt. Chem.*, [2] **32**, 65 (1885).

Kjeldahl: 0.3784 g. subst.; 13.10 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{10}O_4NBr$: N, 4.86%. Found: N, 4.85%.

2-Bromo-4-aminophenoxyacetic Acid.—The acetamino acid was hydrolyzed with 1:1 hydrochloric acid, the amino acid hydrochloride separating from the solution on cooling. This was converted into the free amino acid by treatment with sodium acetate in the usual way and the amino acid purified by a repetition of the process. The acid forms minute, almost colorless prisms which are very sparingly soluble in the usual neutral solvents. When rapidly heated it melts with effervescence at about $230-5^{\circ}$, resolidifying immediately to a mass which does not melt below 280° ; if the heating is carried out slowly, the substance merely sinters above 215° and does not melt below 280° . An aqueous suspension gives a violet color with ferric chloride.

Kjeldahl: 0.3663 g. subst.; 14.60 cc. 0.1 *N* HCl.

Calc. for $C_8H_8O_3NBr$: N, 5.69%. Found: N, 5.58%.

The Methyl Ester Hydrochloride.—This salt was obtained from the amino acid hydrochloride by esterification in the usual way. Recrystallized from absolute alcohol, it forms glistening platelets which soften above 210° and melt with effervescence at $220-2^{\circ}$. The salt is quite readily soluble in water, the solution giving a slowly developing wine-red color with ferric chloride.

0.3173 g. subst.; 0.1540 g. AgCl.

Calc. for $C_9H_{10}O_3NBr.HCl$: Cl, 11.95%. Found: Cl, 12.01%.

The Methyl Ester.—The base, obtained by decomposition of the salt with dilute sodium carbonate, was recrystallized first from absolute alcohol, then from an insufficient amount of hot ligroin (b. p. $90-100^{\circ}$), the colored impurities remaining in the insoluble portion. The ester forms long, delicate, glistening needles which soften at 73.5° and melt at 74.5° (cor.). It is readily soluble in benzene, ether, or methyl alcohol.

0.1839 g. subst.; 8.6 cc. N, 761 mm., 20.0° .

Calc. for $C_9H_{10}O_3NBr$: N, 5.39%. Found: N, 5.33%.

(11) *2-Methyl-4-amino-6-bromophenoxyacetic Acid*.

2-Methyl-4-acetamino-6-bromophenol Hydrobromide.—This unusual salt was obtained as follows: 30 g. *p*-acetamino-*o*-cresol were dissolved in 300 cc. of hot acetic acid and cooled to about 35–40°. To the supersaturated solution was added, drop by drop, with constant stirring, a solution of 9.2 cc. bromine in 3 volumes of acetic acid. The hydrobromide separated when the solution was cooled and scratched, the yield being 44.5 g. Recrystallized from acetic acid containing a little hydrobromic acid the salt forms pale cream-colored crystalline aggregates which soften slightly above 180° and melt at 194–6° with effervescence.

0.1643 g. subst.; 9.6 cc. AgNO₃ soln.¹

Calc. for C₉H₁₀O₂NBr.HBr: Br⁻, 24.58%. Found: Br⁻, 24.49%.

*2-Methyl-4-acetamino-6-bromophenol (2-bromo-4-acetamino-*o*-cresol)*.—A portion of the salt was dissolved in a large volume of boiling water and treated with sodium acetate solution, whereupon the free acetaminobromocresol separated. Recrystallized from toluene, it formed cream-colored, woolly masses of delicate hairs which melted constantly at 155–6° (cor.), with preliminary softening. As Janney² reports, the melting point as 152°, and we were therefore in some doubt as to the position of the bromine on the nucleus, the compound was synthesized as follows:

*2-Bromo-4-amino-*o*-cresol (2-methyl-4-amino-6-bromophenol) Hydrochloride*.—4-Nitro-*o*-cresol was brominated in acetic acid solution according to Auwers³ and the product reduced in the usual way with stannous chloride and hydrochloric acid, filtering off the sparingly soluble double tin salt which separated. The yield of the aminophenol hydrochloride was about 50% of the theory. Kehrman, Mussmann, and Facchinetti⁴ prepared the salt by reducing the bromoquinoneoxime but characterized it incompletely. Recrystallized from warm absolute alcohol by the addition of an equal volume of

¹ 1 cc. = 0.004192 g. Br.

² Janney, *Ann.*, 398, 354 (1913).

³ Auwers, *Ber.*, 39, 3174 (1906).

⁴ Kehrman, Mussmann and Facchinetti, *Ber.* 48, 2021 (1915).

dry ether, the salt forms minute, flat needles which darken above 230° and decompose at $265-70^{\circ}$. It gives a purple color with ferric chloride.

Kjeldahl: 0.2048 g. subst.; 8.6 cc. 0.1 *N* HCl.

Calc. for $C_7H_5ONBr \cdot HCl$: N, 5.87%. Found: N, 5.88%.

The free base obtained from the above salt melted at $146-8^{\circ}$, as found by Janney, and not as given by the above authors. When the amino hydrochloride is acetylated as in previous cases 2-methyl-4-acetamino-6-bromophenol is formed. The analysis, melting point, and mixed melting point determinations showed it to be identical with the substance obtained by brominating *p*-acetamino-*o*-cresol as above.

Kjeldahl: 0.3701 g. subst.; 15.0 cc. 0.1 *N* HCl.

Carius: 0.1451 g. subst.; 0.1133 g. AgBr.

Calc. for $C_9H_{10}O_2NBr$: N, 5.74%; Br, 32.74%. Found: N, 5.68%; Br, 33.24%.

2-Methyl-4-acetamino-6-bromophenoxyacetic Acid.—The above 2-bromo-4-acetamino-*o*-cresol hydrobromide was used as starting material for this substance. One equivalent of the salt, one of chloroacetic acid, and three of sodium hydroxide were boiled in aqueous solution until neutral. The acetamino acid separated on acidifying to Congo red. Recrystallized twice from acetic acid, it forms minute, cream-colored, interlaced needles, which melt at $216-6.5^{\circ}$ (cor.) with preliminary softening. It is somewhat less sparingly soluble in acetone or absolute alcohol than in the other usual solvents.

Kjeldahl: 0.3015 g. subst.; 10.15 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{12}O_4NBr$: N, 4.64%. Found: N, 4.72%.

The acid was also prepared from a portion of the bromoacetamino-cresol obtained from the nitro compound and proved identical in every way to the substance just described.

2-Methyl-4-amino-6-bromophenoxyacetic Acid.—The acetamino acid was boiled under a reflux condenser with 5 parts of 1:1 hydrochloric acid for about 15 minutes, until the amino acid hydrochloride began to separate from the boiling solution. The salt was converted into the free acid in the usual manner and this purified by a repetition of the process. The acid forms thin, almost colorless platelets, which melt with decomposition at 223° . It is very difficultly soluble in the

usual solvents with the exception of boiling alcohol. An aqueous suspension gives no color with ferric chloride in the cold, but on boiling the liquid turns dark brown, soon changing to a lighter red brown.

Kjeldahl: 0.3058 g. subst.; 11.60 cc. 0.1 *N* HCl.

Calc. for $C_9H_{10}O_3NBr$: N, 5.39%. Found: N, 5.31%.

The Methyl Ester Hydrochloride.—The salt separated from the reaction mixture after saturating a suspension of the amino acid in dry methyl alcohol with hydrochloric acid gas and cooling the resulting solution. Recrystallized from methyl alcohol, it forms practically colorless, interlaced needles, which dissolve rather sparingly in water. When rapidly heated, the salt melts and decomposes at 245–50° with preliminary darkening and softening. Its aqueous solution gives a slowly-developing rose color with ferric chloride.

0.2758 g. subst.; 0.1270 g. AgCl.

Calc. for $C_{10}H_{12}O_3NBr.HCl$: Cl, 11.42%. Found: Cl, 11.39%.

The Methyl Ester.—The base separated as an oil which soon solidified. Recrystallized first from 50% alcohol, then from toluene by adding ligroin to the warm solution until just turbid, it forms sheaves of long needles which soften above 57° and melt at 59° (cor.). The ester is readily soluble in the usual organic solvents with the exception of ligroin.

Kjeldahl: 0.3395 g. subst.; 12.55 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{12}O_3NBr$: N, 5.11%. Found: N, 5.18%.

(12) 4-Amino-6-methoxyphenoxyacetic Acid.

In our first efforts to prepare this acid recourse was had to the reaction between nitrophenol sodium salts and chloroacetic ethyl ester. Although no difficulty had been experienced in the case of the sodium salt of 5-nitroguaiacol it was found that the dried salt of the 4-nitro isomer failed to react, even in the presence of sodium iodide as catalyzer. The acetyl derivative of the desired amino acid was synthesized, however, by heating 4-acetaminoguaiacol¹ in aqueous solution with chloroacetic acid and alkali in the usual manner. The acet-amino acid was then readily hydrolyzed to the amino compound.

¹ From the aminoguaiacol as in previous examples.

4-Acetamino-6-methoxyphenoxyacetic Acid.—8 g. 4-acetaminoguaiacol, 4.2 g. chloroacetic acid, 3.6 g. sodium hydroxide, and 40 cc. water were boiled for about one hour. The sodium salt of the acid separated on cooling. The mixture was diluted with water and acidified with hydrochloric acid. The yield of acetamino acid was about 85% of the theory. Recrystallized first from water, then acetic acid, it forms almost colorless nodules of microscopic crystals which melt at about $190-1^{\circ}$, the point of fusion depending somewhat on the rate of heating. The acid is sparingly soluble in the cold in the usual neutral solvents.

Kjeldahl: 0.3839 g. subst.; 15.9 cc. 0.1 N HCl.

Calc. for $C_{11}H_{13}O_5N$: N, 5.86%. Found: N, 5.80%.

4-Amino-6-methoxyphenoxyacetic Acid.—A batch of the acetamino acid obtained by working up to 60 g. 4-aminoguaiacol was boiled under a reflux condenser with about 500 cc. 1:1 hydrochloric acid for about one hour. The solution was evaporated *in vacuo*, taken up with water, and carefully neutralized with sodium carbonate solution, the amino acid separating on scratching. The yield was 60 g. Recrystallized from water it forms almost colorless, glistening plates which are less sparingly soluble in boiling water than in boiling alcohol or acetone. An aqueous suspension gives a deep purple color with ferric chloride. When rapidly heated the acid melts at 190° with decomposition.

0.1640 g. subst.; 10.8 cc. N, 761 mm., 21.5° .

Calc. for $C_9H_{11}O_4N$: N, 7.11%. Found: N, 7.45%.

The Ethyl Ester Hydrochloride.—The salt was prepared as in analogous cases already described, separated from the reaction mixture on cooling. Recrystallized from absolute alcohol it forms delicate, felted needles which melt at $180-6^{\circ}$ with preliminary softening. It is readily soluble in the cold in water or methyl alcohol, less easily in absolute alcohol. The aqueous solution gives a purple color with ferric chloride and diazotizes readily, coupling with R-salt to give a dye of a deeper red shade than those obtained with most of the other aminophenoxyacetic acids. The free ester was obtained by adding aqueous sodium carbonate to the salt, but did not crystallize.

0.2549 g. subst.; 0.1415 g. AgCl.

Calc. for $C_{11}H_{13}O_4N.HCl$: Cl, 13.56%. Found: Cl, 13.73%.

(13) *4-Amino-6-carboxyphenoxyacetic Acid*.

In this case the synthesis of the desired amino acid was rendered difficult not only by the fact that the dried sodium salt of 5-nitrosalicylic methyl ester failed to react with ethyl chloroacetate, but also by the unsatisfactory result of an attempt to react 5-acetaminosalicylic acid with chloroacetic acid and alkali in aqueous solution. The amino acid was finally synthesized, however, by the following series of reactions:

4-Nitro-6-aldehydophenoxyacetic Acid.—5 g. *o*-aldehydophenoxyacetic acid were added in portions to 25 g. fuming nitric acid (d. 1.52), keeping the temperature below 5°. The clear, yellow solution was allowed to stand for 2–3 minutes and was then poured onto ice, precipitating the nitro derivative in excellent yield. Recrystallized first from acetic acid, then ethyl acetate, it forms colorless, microscopic rhombs which melt at 190–2° (cor.) with preliminary softening. In the cold it is somewhat soluble in acetone or ethyl acetate, sparingly in alcohol or acetic acid, and almost insoluble in water. It dissolves sparingly in hot water to a colorless solution, which turns yellow on adding sodium hydroxide. The position of the nitro group was proven after oxidation to the corresponding carboxylic acid (see below).

0.1810 g. subst.; 9.6 cc. moist N, 756 mm., 19.4°.

Calc. for $C_9H_7O_6N$: N, 6.22%. Found: N, 6.02%.

The Acid Phenylhydrazone.—Equimolecular amounts of the acid and phenylhydrazine were warmed in 50% alcohol for one-half hour on the water bath. A yellow color developed immediately and the acid was converted into an orange precipitate. This was filtered off and recrystallized from 85% alcohol, forming glistening, brown-orange rhombs, which dissolved very sparingly in benzene, chloroform, or cold acetic acid, and more readily in acetone or ethyl acetate. When rapidly heated to about 220° and then slowly, it decomposes at 222°. The phenylhydrazone is very resistant to hydrolysis by boiling aqueous alcoholic hydrochloric acid, dissolves in sulfuric acid with an orange-red color, and yields a difficultly soluble, orange-red sodium salt with dilute aqueous sodium hydroxide or carbonate.

0.1375 g. subst.; 15.9 cc. moist N, 755 mm., 21.0°.

Calc. for $C_{15}H_{13}O_6N_3$: N, 13.33%. Found: N, 13.02%.

4-Nitro-6-carboxyphenoxyacetic Acid.—4.9 g. of the nitroaldehyde acid were dissolved in 60 cc. water containing 2.4 g. sodium carbonate and to the solution was slowly added a warm 4% solution of potassium permanganate until a permanent pink color was obtained. About 60 cc. of the permanaganate solution were required. The mixture was decolorized with alcohol, filtered, and the filtrate acidified with sulfuric acid. The yield was excellent. Recrystallized from water, the acid forms warty aggregates of very faintly yellow, microscopic hairs which appear brownish under the microscope. When rapidly heated to 230° and then slowly, it turns yellow at about 235° and melts with decomposition at 238–40°. It is very sparingly soluble in the usual solvents, the solution in hot water having a light yellow color.

0.1519 g. subst.; 7.95 cc. moist N, 752 mm., 21.3°.

Calc. for $C_9H_7O_7N$: N, 5.81%. Found: N, 5.85%.

The position of the nitro group was determined by boiling a portion of the acid for one hour with a saturated solution of hydrobromic acid in acetic acid. The solution was evaporated to small bulk, taken up with water, and the precipitate twice recrystallized from water. In its properties it corresponded exactly with 5-nitrosalicylic acid, and its identity with this was futher shown by a mixed melting point with a sample prepared by nitration of salicylic acid, no depression being caused.

4-Nitro-6-carbethoxyphenoxyacetic Ethyl Ester.—3.4 g. of the above acid were suspended in 13.6 g. absolute alcohol and boiled 3.5–4 hours under a reflux condenser after adding 6.8 g. of concentrated sulfuric acid. The ester crystallized on cooling and was filtered off. Recrystallized from 95% alcohol it forms delicate, glistening, unctuous needles, which melt at 75–6° (cor.). It is sparingly soluble in the cold in 95% alcohol or ligroin, somewhat more soluble in absolute alcohol, and easily in the other organic solvents.

Kjeldahl: 0.3121 g. subst.; 9.6 cc. 0.1 N HCl.

Calc. for $C_{13}H_{15}O_7N$: N, 4.71%. Found: N, 4.31%.

4-Amino-6-carboxyphenoxyacetic Acid.—This was obtained by reduction of the nitro acid or nitro ester in alcoholic solution with tin and hydrochloric acid, the usual manipulations being followed. The

amino acid was liberated from the hydrochloride in the usual way and purified by solution in dilute hydrochloric acid, precipitation with sodium acetate, and then dissolving in dilute sodium hydroxide and precipitating with acetic acid. The substance forms pale brown aggregates of plates which decompose slightly, but do not melt below 280° . It is very difficultly soluble in the usual neutral solvents and, in aqueous suspension, gives a light red-brown color with ferric chloride.

0.1617 g. subst.; 8.8 cc. N, 755 mm., 22.0° .

Calc. for $C_9H_9O_5N.H_2O$: N, 6.11%. Found: N, 6.09%.

The Ethyl Ester Hydrochloride.—One part of the amino acid hydrochloride, 2 parts of concentrated sulfuric acid, and 4 parts of absolute alcohol were heated under a reflux condenser for about 3.5 hours. Water and ice were then added and the solution made alkaline to phenolphthalein and immediately extracted with ether. The extract was dried over sodium sulfate, evaporated to dryness, and the residue taken up in absolute alcohol saturated with hydrochloric acid, precipitating the ester salt. After addition of dry ether the salt was filtered off. Recrystallized from absolute alcohol, it forms aggregates of delicate needles which soften at about 140° , become transparent at 146° , and are completely molten at $156-7^{\circ}$. The salt is slowly, although freely soluble in water, the solution giving a yellow brown color, darkening to red-brown, with ferric chloride.

0.2701 g. subst.; 16.45 cc. $AgNO_3$ Soln.¹

alc. for $C_{13}H_{17}O_5N.HCl$: Cl, 11.68%. Found: Cl, 11.33%.

The Ethyl Ester.—The ester was obtained from the hydrochloride as an oil which solidified in the ice box. It was recrystallized twice from absolute alcohol with the aid of a freezing mixture, forming slightly brownish crystals, which melted at $74-6^{\circ}$ (cor.) with preliminary softening and dissolved less readily in ligroin and ether than in the other usual organic solvents.

0.2002 g. subst.; 9.6 cc. N, 763 mm., 26.0° .

Calc. for $C_{13}H_{17}O_5N$: N, 5.24%. Found: N, 5.32%.

¹ 1 cc. = 0.00186 g. Cl.

(14) *4-Amino-6-acetophenoxyacetic Acid.*

4-Acetamino-6-acetophenoxyacetic Acid.—22 g. 2-hydroxy-5-acetaminoacetophenone, 10.7 g. chloroacetic acid, 18.5 g. sodium hydroxide, and 200 cc. water were boiled down to small bulk in an open flask, repeating the process with one-half the amounts of chloroacetic acid, alkali, and water. After diluting and acidifying with acetic acid the mixture was allowed to stand overnight. A small amount of unchanged hydroxyacetaminoacetophenone was filtered off and the new acid precipitated from the filtrate by the addition of hydrochloric acid. The yield was 23.6 g. Recrystallized twice from acetic acid, the substance forms faintly greenish gray, woolly masses of delicate needles which are soluble in hot 95% alcohol or acetic acid and only sparingly soluble in hot water. When rapidly heated to 220° and then slowly the acid softens, then melts to a brown liquid and evolves gas at 223–6°. It dissolves in concentrated sulfuric acid with an olive-yellow color.

Kjeldahl: 0.3374 g. subst.; 13.40 cc. 0.1 N HCl.

Calc. for $C_{12}H_{13}O_5N$: N, 5.58%. Found: N, 5.56%.

4-Amino-6-acetophenoxyacetic Acid.—The acetamino acid was hydrolyzed by heating with 1:1 hydrochloric acid. The amino acid hydrochloride separated on cooling. A portion of this was dissolved in warm water and converted into the free acid by adding sodium acetate. Recrystallized from water, it forms long, pale brown, glistening needles, which contain between 1 and 1.5 molecules of water of crystallization. When anhydrous and rapidly heated, it darkens above 125° and gradually softens to a tar which decomposes at about 145°. The acid is soluble in hot water or hot absolute alcohol, and, in aqueous suspension, gives a slowly-developing purple color with ferric chloride. The diazonium solution couples with R-salt to give a deeper red color than the shades given by most of the other aminophenoxyacetic acids.

0.9402 g. subst., air-dry, lost 0.0860 g. H_2O , 9.15%. Calc.: 1 H_2O , 7.93%; 1.5 H_2O , 11.44%.

Recrystd.: 0.3551 g. subst., air-dry, lost 0.0359 g. H_2O , 10.11%.

Kjeldahl: 0.3107 g. subst., anhydrous; 15.0 cc. 0.1 N HCl.

Calc. for $C_{10}H_{11}O_4N$: N, 6.70%. Found: N, 6.76%.

4-Amino-6-acetophenoxyacetic Methyl Ester Hydrochloride.—This substance was prepared from the crude amino acid hydrochloride as in numerous preceding examples. Separation of the salt from the reaction mixture was completed by the addition of dry ether. Recrystallized from absolute alcohol containing a drop of concentrated hydrochloric acid, the hydrochloride forms nacreous, diamond-shaped plates which dissolve readily in water, less easily in absolute alcohol. When rapidly heated to 190° and then slowly it darkens above this point and melts with decomposition at $204-7^{\circ}$.

0.3090 g. subst.; 0.1681 g. AgCl.

Calc. for $C_{11}H_{13}O_4N.HCl$: Cl, 13.67%. Found: Cl, 13.46%.

The Methyl Ester.—The free base was obtained by the action of sodium carbonate on an aqueous solution of the salt. Recrystallized from $95^{\circ}C$ alcohol, the ester forms flat, pale yellow needles, which melt at $141-2.5^{\circ}$ (cor.) with slight preliminary softening. It is sparingly soluble in the cold in alcohol or benzene, soluble in acetone. An aqueous suspension gives a slowly-developing wine-red color with ferric chloride.

Kjeldahl: 0.2988 g. subst.; 13.35 cc. 0.1 N HCl.

Calc. for $C_{11}H_{13}O_4N$: N, 6.28%. Found: N, 6.26%.

(15) *Amino-o-phenylenedi-[oxyacetic Acid]*.

Nitro-o-phenylenedi-[oxyacetic Acid].—40 g. *o*-phenylenedioxyacetic acid were added in portions to 160 cc. of concentrated nitric acid, keeping the temperature at about 25° . The phenoxyacetic acid slowly dissolved. After letting stand for about two hours the solution was poured into a large volume of water. The precipitated nitro acid was recrystallized from water, separating in a yield of 40.5 g. Recrystallized again from water, then from acetic acid, it forms practically colorless aggregates of minute crystals which melt at $181-3^{\circ}$ (cor.) with preliminary softening. The acid separates from water with one molecule of water of crystallization and is sparingly soluble in the cold in the usual solvents.

2.4361 g. subst.; 0.1552 g. loss. H_2O , 6.37%. $1 H_2O$, 6.23%.

Anhydrous: 0.2053 g. subst.; 10.15 cc. N, 752 mm., 20.4° .

Calc. for $C_{10}H_8O_3N$: N, 5.17%. Found: N, 5.56%.

In attempts to fix the position of the nitro group the acid was boiled 1.5 hours with a saturated solution of hydrobromic acid in acetic acid, but was recovered unchanged. When heated in a sealed tube at 155–65° most of the substance was carbonized, only a small amount of a soluble hydrobromide being recovered. It seems reasonable to suppose, however, that the nitro group is in Position 4.

Amino-o-phenylenedi-[oxyacetic Acid].—The nitro acid was reduced in hot alcoholic solution with tin and concentrated hydrochloric acid until a test portion remained clear on diluting with water. The amino acid hydrochloride was isolated in the usual way and converted into the free acid by dissolving in water and adding sodium acetate. The acid separated on scratching, 17 g. being obtained from 40.5 g. of the nitro acid. Purified by dissolving in dilute hydrochloric acid and reprecipitating with sodium acetate the substance forms grayish microcrystals which, when rapidly heated, darken above 240° and decompose at 243–5°. It is sparingly soluble in the usual neutral solvents, and, in aqueous suspension, gives a violet color with ferric chloride.

Kjeldahl: 0.3209 g. subst.; 13.15 cc. 0.1 N HCl.

Calc. for $C_{10}H_{11}O_6N$: N, 5.81%. Found: N, 5.74%.

(D) *Aminophenoxyacetic Acids with Condensed Nuclei.*

(1) *4-Amino-1-naphthoxyacetic Acid.*

4-Acetamino-1-naphthoxyacetic Acid.—4-Acetamino-1-naphthol was prepared from the aminonaphthol hydrochloride by the action of acetic anhydride in aqueous solution, followed by sodium acetate, as in previous examples. When boiled in aqueous solution with chloroacetic acid and sodium hydroxide, repeating the treatment with one-half the original amounts, it gave a practically quantitative yield of the acetaminonaphthoxyacetic acid. Recrystallized twice from acetic acid, the substance formed practically colorless aggregates of spears which softened above 225° and melted at 233–4°. The acid is very difficultly soluble in the cold in the usual solvents, dissolving, however, in hot acetic acid or absolute alcohol, and slightly in hot acetone.

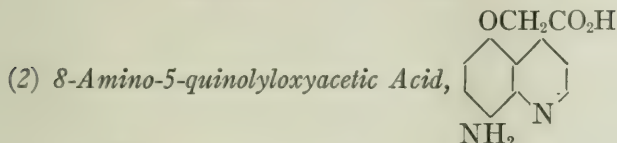
Kjeldahl: 0.3081 g. subst.; 11.70 cc. 0.1 N HCl.

Calc. for $C_{14}H_{13}O_4N$: N, 5.41%. Found: N, 5.32%.

4-Amino-1-naphthoxyacetic Acid.—The amino acid was obtained by boiling the acetamino compound with 1:1 hydrochloric acid as in previous examples and liberating the free acid from the salt by means of sodium acetate. Recrystallized from boiling amyl acetate, in which it is less sparingly soluble than in the other neutral solvents, the amino acid forms radiating masses of minute needles which turn purple above 215° and melt with decomposition at 220–4°. In aqueous suspension it gives a blue color with ferric chloride, and, in sodium carbonate solution, couples readily with benzenediazonium chloride, yielding a red precipitate. On adding sodium nitrite to a solution of the amino acid in dilute hydrochloric acid a blue color is produced owing to partial oxidation, but the diazonium solution formed couples readily with R-salt to give a purple color.

Kjeldahl: 0.2946 g. subst.; 13.40 cc. 0.1 *N* HCl.

Calc. for $C_{12}H_{11}O_3N$: N, 6.45%. Found: N, 6.37%.



8-Acetamino-5-hydroxyquinoline.—8-Amino-5-hydroxyquinoline sulfate was prepared by the method of electrolytic reduction worked out by Gattermann.¹ The salt was suspended in 10 parts of water, treated with 1.1 mols of acetic anhydride, and rapidly turbinized while adding strong sodium acetate solution. The salt quickly dissolved and the acetyl derivative separated in shining, greenish leaflets. In order to saponify any diacetyl compound that may have been formed sodium hydroxide was added to the mixture in slight excess, stirring until solution was complete. After reprecipitating the acetyl derivative with acetic acid, the mixture was cooled and the product filtered off and washed with water. The yield was practically quantitative. Recrystallized twice from 85% alcohol, the substance forms practically colorless leaflets that turn green on exposure to light and air. It decomposes partially above 190°, melting to a turbid, brown liquid at 221–3° and clearing completely at 227°. It is sparingly

¹ Gattermann, *Ber.*, 27, 1940 (1894)

soluble in the cold in water or acetic acid, and very difficultly so in acetone or benzene. It dissolves with difficulty in dilute hydrochloric acid with an orange color, in dilute sodium hydroxide with an olive color. An aqueous suspension gives a deep olive color with ferric chloride.

0.1348 g. subst.; 16.2 cc. N, 767 mm., 25.0°.

Calc. for $C_{11}H_{10}O_2N_2$: N, 13.87%. Found: N, 13.89%.

8-Acetamino-5-quinolyloxyacetic Acid.—The acetaminohydroxyquinoline was condensed with chloroacetic acid in the same way as the preceding naphthyl compound. The new acid was isolated by adding concentrated hydrochloric acid to the reaction mixture, precipitating the hydrochloride as a thick mass of glistening needles. The mixture was cooled in ice-water and filtered, washing the salt with saturated sodium chloride solution. The product was then dissolved in water and treated with sodium acetate until neutral to Congo red. The yield of free acid obtained in this way from 30.3 g. of the acetaminohydroxyquinoline was 32 g. The acid separates from boiling water as brownish, glistening leaflets containing about one molecule of water of crystallization. When rapidly heated it decomposes slightly and softens, finally melting with gas evolution at 255°. It dissolves in dilute hydrochloric acid or sodium hydroxide to give pale greenish yellow solutions.

1.0257 g. subst.; 0.0750 g. loss. H_2O , 7.31%. 1 H_2O , 6.47%.

Anhydrous: 0.1031 g. subst.; 9.7 cc. N, 751 mm., 21.0°.

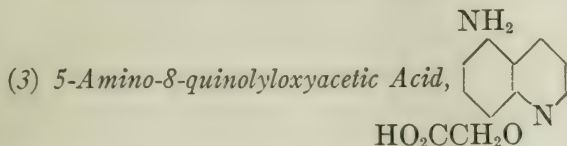
Calc. for $C_{13}H_{12}O_4N_2$: N, 10.77%. Found: N, 10.80%.

8-Amino-5-quinolyloxyacetic Acid.—The acetamino acid was hydrolyzed as in previous cases, the amino acid hydrochloride separating on chilling. A further quantity was obtained by concentrating the filtrate *in vacuo*. When dissolved in water and neutralized with sodium acetate the salt yielded the free acid, 20 g. being obtained from 25 g. of the acetamino compound. A portion of the hydrochloride was recrystallized from 10% hydrochloric acid and the free acid liberated from this and recrystallized by dissolving in warm, very dilute hydrochloric acid and again adding sodium acetate solution. It forms brown, glistening crystals which soften above 190° when rapidly heated and melt and decompose at about 225°. When slowly heated the

acid gradually softens above 190° to a tar which does not melt completely below 275° . It is readily diazotized, giving a purple color with R-salt, and, in aqueous suspension, gives a deep red-brown color with ferrid chloride.

0.1028 g. subst.; 11.0 cc. N, 747 mm., 17.5° .

Calc. for $C_{11}H_{10}O_3N_2$: N, 12.84%. Found: N, 12.36%.



5-Amino-8-hydroxyquinoline Dihydrochloride.—Lippmann and Fleissner¹ reduced 5-nitroso-8-hydroxyquinoline hydrochloride with stannous chloride and hydrochloric acid, converting the amino hydrochloride into the free base. The nitroso compound may be very conveniently reduced by dissolving in ammonia and passing in hydrogen sulfide. 70 g. 5-nitroso-8-hydroxyquinoline hydrochloride were dissolved as well as possible in 1050 cc. of dilute ammonia (one part of concentrated to 1.5 parts of water) and the solution saturated with hydrogen sulfide. The precipitate of amino compound was filtered off, washed with water, suspended in a little water, treated with a slight excess of hydrochloric acid, and shaken until the base had dissolved. The solution was then filtered from the residue of sulfur and the dihydrochloride precipitated by the addition of concentrated hydrochloric acid or by passing in a stream of hydrochloric acid gas until saturated. After filtering off the first crop of crystals a further quantity was obtained by concentrating the filtrate to small bulk *in vacuo*. The total yield was 40 g. Recrystallized from 10% hydrochloric acid solution it forms crusts of brown plates which do not melt sharply when heated, but gradually soften and decompose, markedly at about 245° , but not melting completely below 280° . The salt dissolved in dilute hydrochloric acid with a yellow color, in water with an orange-red color, and in dilute sodium hydroxide with an olive color. The aqueous solution turns brown on adding ferric chloride.

¹ Lippmann and Fleissner. *Monatsh.*, 10, 796 (1889).

Carius: 0.1171 g. subst.; 0.1414 g. AgCl.

Calc. for $C_9H_8ON_2 \cdot 2HCl$: Cl, 30.41%. Found: Cl, 29.88%.

5-Acetamino-8-hydroxyquinoline.—38 g. of the above dihydrochloride were dissolved in 200 cc. water, treated with 25 cc. acetic anhydride, and then, with vigorous turbinig, with an excess of sodium acetate. The reaction occurred at once, forming a dark-colored solution. The acetamino compound was precipitated from this by adding an excess of ammonia, separating gradually as greenish gray, lenticular plates. After recrystallizing from water the yield was 18.5 g. Recrystallized first from water, using bone-black, then from 95% alcohol, the substance forms almost colorless prisms which melt at $221-2^\circ$ (cor.) with preliminary softening. It is insoluble in benzene, acetone, cold 95% alcohol or water, and dissolves in acetic acid with an orange-yellow color, in dilute alkalis and acids with a greenish yellow color, and in aqueous suspension gives a deep blue-green coloration with ferric chloride.

0.1381 g. subst.; 16.8 cc. N, 763 mm., 26.0° .

Calc. for $C_{11}H_{10}O_2N_2$: N, 13.87%. Found: N, 13.94%.

5-Acetamino-8-quinolyloxyacetic Acid.—17 g. 5-acetamino-8-hydroxyquinoline, 8 g. chloroacetic acid, 13.5 g. 50% aqueous sodium hydroxide, and about 170 cc. water were slowly boiled down to small volume. One-half of the above quantities of chloroacetic acid, alkali, and water were then added and the solution again boiled down to small bulk. The resulting liquid was diluted with water and acidified faintly to Congo red with hydrochloric acid, the acetaminoquinolyloxyacetic acid separating on chilling as a voluminous mass of long, thin, yellow needles. On concentrating the filtrate a further crop was obtained, the total yield being 19.7 g. As isolated in this manner, the acid was sufficiently pure for conversion into the amino compound as described below, but could not be freed from its content of hydrochloric acid by simple recrystallization, even in the presence of sodium acetate. An analytically pure specimen was finally obtained as follows: A portion of the crude acid was dissolved in dilute sodium hydroxide and the solution treated with a slight excess of acetic acid. A product separated in which halogen still persisted. This was therefore dissolved in hot water, rapidly cooled, and treated with about one-half volume

of 10% aqueous nitric acid. 5-Acetamino-8-quinolyloxyacetic acid nitrate soon separated in voluminous masses of yellow hairs. The salt was filtered off and dried *in vacuo* at room temperature over sulfuric acid, after which it darkened and softened at 150–5° and decomposed at 225–30°. It is soluble in water or hot 95% alcohol.

0.1052 g. subst.; 11.85 cc. N, 765 mm., 24.5°.

Calc. for $C_{13}H_{12}O_4N_2 \cdot HNO_3$: N, 13.00%. Found: N, 13.02%.

The nitrate was dissolved in a little hot water and the solution treated with an excess of sodium acetate. On standing in the ice box the free acid gradually separated as orange prisms and rhombs which were halogen-free and contained between two and two and half molecules of water of crystallization. On drying to constant weight at 100° *in vacuo* over sulfuric acid the substance lost its water and became straw-colored. When rapidly heated to 250°, then slowly, it softened and then melted with decomposition at 253–5°. The anhydrous acid is almost insoluble in dry acetone or absolute alcohol, and turns bright yellow under water, dissolving somewhat sparingly on heating.

Air-dry: 0.3392 g. lost 0.0467 g. H_2O , 13.77%. Calc.: 2 H_2O , 12.17%; 2.5 H_2O , 14.76%.

Anhydrous: 0.1468 g. subst.; 14.2 cc. N, 756 mm., 27.5°.

Calc. for $C_{13}H_{12}O_4N_2$: N, 10.77%. Found: N, 10.93%.

The Acid Dihydrochloride.—The crude acetamino acid was hydrolyzed by boiling with 5 parts of 1:1 hydrochloric acid for 2 hours. Most of the amino acid salt separated on cooling and scratching, a further quantity being obtained by concentrating the filtrate *in vacuo*. Recrystallized from a small volume of 10% aqueous hydrochloric acid, the salt formed long, narrow, brownish plates which turned slightly reddish in color when dried to constant weight *in vacuo* at room temperature over sulfuric acid. When rapidly heated to 150°, then slowly, the salt first reddens, then gradually softens, sinters, and darkens above 155°, and melts with gas evolution to a bright red mass at 160–2°. It is readily diazotized, coupling with R-salt to give a dark purplish red color. It dissolves in water with an orange-red color, changing to deep red on adding ferric chloride. The solution of the salt in dilute hydrochloric acid is brownish yellow, while in

dilute sodium hydroxide the color is pale yellow. When moistened with water the salt turns a deep brown-red, giving a solution of the same color on adding sodium acetate. The solution then gradually deposits a network of dark brown-red needles which dissolve very easily in water and probably consist of the free acid or its sodium salt.

0.1102 g. subst.; 0.1080 g. AgCl.

Calc. for $C_{11}H_{10}O_3N_2 \cdot 2HCl$: Cl, 24.35%. Found: Cl, 24.25%.

The Methyl Ester.—14 g. of the crude hydrochloride were suspended in 140 cc. dry methyl alcohol and esterified by the action of dry hydrochloric acid gas. Separation of the ester dihydrochloride was completed by adding an equal volume of dry ether, the yield being 14 g. The salt forms slightly pinkish, glistening platelets which slowly lose hydrochloric acid and turn red on exposure to moist air. It dissolves in water with a red color which changes to brownish yellow on adding hydrochloric acid. The free ester separated from an aqueous solution of the salt on adding sodium carbonate and was recrystallized from 95% alcohol, forming lemon-yellow rhombs and flat needles which melted, with slight preliminary softening, at $176-7^\circ$ (cor.). It is sparingly soluble in benzene or cold 95% alcohol but dissolves in acetone. An acetic acid solution of the ester has a red color, while an aqueous suspension gives an orange color with ferric chloride. It readily diazotized, coupling with R-salt to form a dark red dye.

0.1171 g. subst.; 11.45 cc. N, 766 mm., 18.0° .

Calc. for $C_{12}H_{12}O_3N_2$: N, 12.07%. Found: N, 11.57%.

(E) *Aminophenoxybutyric Acids.*

(1) *o*-Amino- γ -phenoxybutyric Acid, $o\text{-H}_2\text{NC}_6\text{H}_4\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$.

o-Acetaminophenoxypropyl Bromide (*o*-Acetaminophenyl- γ -bromopropyl Ether, $o\text{-CH}_3\text{CONHC}_6\text{H}_4\text{OCH}_2\text{CH}_2\text{CH}_2\text{Br}$).—34 g. *o*-acetaminophenol were dissolved in 130 cc. alcohol and boiled on the water bath for 2.5 to 3 hours with 18 g. 50% sodium hydroxide solution and 350 g. trimethylene bromide. The alcohol and the excess of trimethylene bromide were then distilled off with steam. The mixture was cooled, made alkaline, and the residual heavy oil removed from the aqueous

solution by shaking out with chloroform. The extract was dried and concentrated, and the oily residue taken up in alcohol. On scratching, a small amount of the propylene ether of *o*-acetaminophenol separated (see below). The amount of propylene ether formed is much larger if a smaller excess of trimethylene bromide is used. The filtrate from the propylene ether was concentrated and the residue treated with ligroin and chilled. The acetaminophenoxypropyl bromide crystallized on rubbing and was filtered off, washed with ligroin, and sucked dry. The yield was 30 g. A portion was purified for analysis by dissolving in a small volume of absolute alcohol bone-blackening, and filtering from a slight residue. The filtrate was diluted with water, giving an oily product which solidified when seeded. This was dried and recrystallized from a small volume of absolute alcohol with the aid of a freezing mixture. The bromide forms snowy, felted needles which soften above 60° and melt at 62–2.5° (cor.). It is readily soluble at room temperature in the usual organic solvents with the exception of ligroin.

0.1357 g. subst.; 6.3 cc. N, 762 mm., 25.5°.

Calc. for $C_{11}H_{14}O_2NBr$: N, 5.15%. Found: N, 5.16%.

Propylene bis- (o-Acetaminophenyl) Ether, $o\text{-CH}_3\text{CONHC}_6\text{H}_4\text{OCH}_2\text{CH}_2\text{CH}_2\text{OC}_6\text{H}_4\text{NHCOCH}_3$.—The alcohol-insoluble product obtained in the preparation of the acetaminophenoxypropyl bromide was recrystallized twice from acetic acid, forming rosetts of prismatic needles which soften at 192.5° and melt at 193.5–4.5° (cor.). The ether is sparingly soluble in the cold in the usual solvents, but is quite soluble in boiling alcohol or chloroform, and readily so in boiling acetic acid.

Kjeldahl: 0.3050 g. subst.; 17.65 cc. 0.1 N HCl.

Calc. for $C_{19}H_{22}O_4N_2$: N, 8.19%. Found: N, 8.11%.

o-Acetaminophenoxypropyl Cyanide (o-Acetaminophenyl- γ -cyanopropyl Ether, o-Acetaminophenoxybutyronitrile).—40 g. of the crude bromide were dissolved in 200 cc. of absolute alcohol and heated to boiling on the water bath. To the boiling solution was added, drop by drop, a solution of 12 g. potassium cyanide in 25 cc. water. After 4 hours' boiling most of the alcohol was evaporated off and the residue treated with water. An oil separated, which soon crystallized. The

substance was filtered off, washed with water, and taken up in a small volume of hot alcohol. On chilling, the solution set to a thick, crystalline mass, which was filtered off and washed with alcohol and ether. 20 g. of the cyanide were obtained in the main fraction, and a further quantity on concentrating the alcoholic filtrate and washings. Recrystallized from toluene, the nitrile forms thick, pale cream-colored, diamond-shaped plates which soften at 88° and melt at $89-90^{\circ}$ (cor.). It is sparingly soluble in the cold in toluene or absolute alcohol and dissolves very readily in chloroform.

Kjeldahl: 0.2041 g. subst.; 18.6 cc. 0.1 N HCl.

Calc. for $C_{12}H_{14}O_2N_2$: N, 12.84%. Found: N, 12.77%.

o-Amino- γ -phenoxybutyric Acid Hydrochloride.—15 g. *o*-acetaminophenoxypropyl cyanide were boiled for 2 hours with 75 cc. 1:1 hydrochloric acid. The amino acid hydrochloride separated as a thick mass on cooling. This was filtered off and washed first with a little 1:1 hydrochloric acid, then with dry acetone. The yield of salt was 12 g. A portion was recrystallized by dissolving in the minimum amount of cold absolute alcohol, adding dry ether until just turbid, and seeding with a crystal. It separates in radiating masses of flat, pointed needles which melt at $180-2^{\circ}$ with slight preliminary softening. The aqueous solution gives a slowly developing purple color with ferric chloride.

0.1596 g. subst.; 12.69 cc. $AgNO_3$ soln.¹

Calc. for $C_{10}H_{13}O_3N.HCl$: Cl, 15.32%. Found: Cl, 14.79%.

o-Amino- γ -phenoxybutyric Acid.—A portion of the hydrochloride was dissolved in a small amount of water and the free acid precipitated as a gum by the addition of sodium acetate solution. The mixture was then shaken with benzene until this had taken up all of the acid. The benzene was dried over sodium sulfate and concentrated to small bulk, whereupon the amino acid gradually crystallized on rubbing and letting stand. It was purified by dissolving in hot benzene, carefully adding petroleum ether until a small fraction containing the colored impurities had separated, adding bone-black, filtering, and precipitating the remainder with petroleum ether as an al-

¹ 1 cc. = 0.00186 g. Cl.

most colorless oil which solidified to a mass of microscopic platelets on seeding and rubbing. The acid melts at $54-7^{\circ}$, with preliminary softening, to a liquid containing bubbles. It dissolves with difficulty in the cold in ligroin or carbon tetrachloride, more easily in toluene or water, and readily in the other usual neutral organic solvents. An aqueous solution gives a slowly developing lilac color with ferric chloride. The acid is readily diazotized, coupling with R-salt to give a deep red dye.

0.1007 g. subst.; 6.6 cc. N, 751 mm., 22.5° .

Calc. for $C_{10}H_{13}O_3N$: N, 7.18%. Found: N, 7.48%.

o-Aminophenoxybutyric Methyl Ester.—15 g. of the amino acid hydrochloride in 130 cc. of dry methyl alcohol were saturated with dry hydrochloric acid gas and allowed to stand for 24 hours. The clear solution was concentrated to dryness *in vacuo* and the residue taken up in water. On adding sodium carbonate solution the ester separated as an oil which rapidly solidified. The yield was 10 g. Recrystallized from ligroin it forms rectangular platelets which melt constantly at $45-5.5^{\circ}$ with slight preliminary softening. It is readily soluble in the usual organic solvents with the exception of ligroin.

Kjeldahl: 0.3601 g. subst.; 16.8 cc. 0.1 N HCl.

Calc. for $C_{11}H_{15}O_3N$: N, 6.70%. Found: N, 6.54%.

(2) *p*-Amino- γ -phenoxybutyric Acid.

p-Acetaminophenoxypropyl Bromide (*p*-Acetaminophenyl- γ -bromopropyl Ether.—This substance was prepared from *p*-acetaminophenol in the same way as the *ortho* isomer. After the steam distillation the residue crystallized on standing overnight, giving a yield of crude product equal to the weight of acetaminophenol used. Recrystallized successively from 95% alcohol, toluene and absolute alcohol, the bromide forms slightly brownish plates which soften above 129° and melt at $133-5^{\circ}$. It is soluble in the cold in acetone or chloroform and sparingly so in alcohol, toluene, or ether.

Kjeldahl: 0.3393 g. subst.; 12.7 cc. 0.1 N HCl.

Calc. for $C_{11}H_{14}O_2NBr$: N, 5.15%. Found: N, 5.24%

p-Acetaminophenoxypropyl Cyanide (*p*-Acetaminophenyl- γ -cyanopropyl Ether, *p*-Acetaminophenoxybutyronitrile).—The cyanide was prepared as in the case of the *ortho* isomer, the yield from 27.3 g. of the bromide being 19.5 g. Recrystallized from absolute alcohol, it melts at 98–100° with preliminary softening. It dissolves readily in alcohol or acetic acid, difficultly in benzene.

Kjeldahl: 0.2957 g. subst.; 27.0 cc. 0.1 *N* HCl.

Calc. for $C_{12}H_{14}O_2N_2$: N, 12.84%. Found: N, 12.79%.

p-Amino- γ -phenoxybutyric Acid Hydrochloride.—After saponification of the nitrile the reaction mixture was cooled to 0° and the salt filtered off. 10 g. of the nitrile yielded 6.3 g. of the hydrochloride. Recrystallized twice from 1:1 hydrochloric acid, using bone-black, it forms flat, grayish prisms which darken above 180° and melt at 191–4° to a brown liquid. It is sparingly soluble in the cold in absolute alcohol and, in aqueous solution, gives a deep violet color with ferric chloride.

0.1828 g. subst.; 0.1125 g. AgCl.

Calc. for $C_{10}H_{13}O_3N.HCl$: Cl, 15.32%. Found: N, 15.22%.

p-Amino- γ -phenoxybutyric Acid.—The free acid crystallized from an aqueous solution of the salt on adding sodium acetate solution. Recrystallized from a small volume of water, using bone-black, the acid forms lustrous, slightly brownish scales which melt at 145.5–6° (cor.) to a brown liquid. It is sparingly soluble in cold alcohol and in hot benzene. The amino acid is readily diazotized, coupling with R-salt to give a deeper red dye than is formed from the simple aminophenoxyacetic acids.

Kjeldahl: 0.3215 g. subst.; 16.45 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{13}O_3N$: N, 7.18%. Found: N, 7.17%.

ON AMIDES, URAMINO COMPOUNDS, AND UREIDES CONTAINING AN AROMATIC NUCLEUS.

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The material for the present paper comprises a number of substances which figured as intermediates in an investigation, the results of which we hope soon to publish. The compounds discussed are amides and ureides of certain purely aromatic acids, amides and ureides of *m*- and *p*-aminophenylacetic acids, amides and ureides of derivatives of various aminophenoxyacetic acids, ureas in which the amide function is also present in the molecule, several sulfonamide derivatives, and one or two aromatic ureas containing less easily classified side chains.

EXPERIMENTAL.

(A) Amides Containing the Aromatic Nucleus.

(1) Amides of Substituted Benzoic Acids.

o-Aminobenzamide.¹

o-Chloroacetylaminobenzamide.²

m-Aminobenzamide.³

m-Chloroacetylaminobenzamide.⁴

p-Aminobenzamide.⁵

p-Chloroacetylaminobenzamide.⁶

¹ THIS JOURNAL, 39, 1437 (1917).

² *Ibid.*, 39, 1442 (1917).

³ *Ibid.*, 39, 1438 (1917).

⁴ *Ibid.*, 39, 1442 (1917).

⁵ *Ibid.*, 39, 1436 (1917), paragraph 2.

⁶ *Ibid.*, 39, 1443 (1917).

5-Aminosalicylamide (3-amino-6-hydroxybenzamide).—This substance was first prepared by reducing *m*-nitrobenzamide according to Gattermann's electrolytic method.¹ 20 g. of the nitro amide were reduced in 150 g. sulfuric acid and then diluted with ice until the volume was doubled. The sulfate of the aminosalicylamide gradually separated on rubbing and was filtered off on alundum. The product was dissolved in water and treated with enough barium chloride solution to precipitate the sulfuric acid. After filtering the solution was concentrated to small bulk *in vacuo*, the hydrochloride crystallizing on cooling. The salt was filtered off and washed with acetone. The yield was 11.5 g. A portion of the salt was dissolved in water and the free base liberated by adding ammonia in slight excess and scratching until crystallization started. Recrystallized from water containing a little ammonia the amide forms almost colorless, silky needles which are difficult to obtain free from color, owing to the tendency of the base to oxidize in solution. When rapidly heated to 190° and then slowly it melts at 194–7° with decomposition. It is sparingly soluble in the cold in water or absolute alcohol, readily in both on heating. It dissolves in acetone but is practically insoluble in benzene. An aqueous suspension of the amide gives a deep brown color with ferric chloride.

Kjeldahl: 0.1519 g. subst.; 19.75 cc. 0.1 *N* HCl.

Calc. for $C_7H_8O_2N_2$: N, 18.43%. Found: N, 18.21%.

In later preparations of the amide it was found more satisfactory, both from the standpoint of convenience and yield, to use 5-amino-salicylic methyl ester as the starting point. 63 g. of the ester were heated in an autoclave at 110° for 6 hours with 130 cc. of concentrated aqueous ammonia. The ammonia was distilled off *in vacuo* and the residue recrystallized from water, decolorizing with bone-black. The amide was obtained in this way in a yield of 45 g. and showed all of the properties given above.

5-Chloroacetylaminosalicylamide.—The aminoamide was chloroacetylated by dissolving in a mixture of 5 parts of saturated sodium acetate solution and 5 parts of acetic acid and adding 1.5 mols of chloroacetyl

¹ Gattermann, *Ber.*, 27, 1927 (1894).

chloride, drop by drop, with stirring and cooling.¹ The resulting solution was concentrated to dryness *in vacuo*, taken up in water, filtered off, and recrystallized from 50% alcohol, using bone-black. The yield was excellent. Recrystallized again, with bone-black, from 85% alcohol the substance forms aggregates of minute, slightly purplish platelets which melt at 225–7° to a dark liquid which slowly evolves gas. It is soluble in hot acetone or boiling water, and is quite soluble in alcohol at room temperature. An aqueous suspension gives a violet color with ferric chloride.

Kjeldahl: 0.1670 g. subst.; 14.60 cc. 0.1 N HCl.

Calc. for $C_9H_9O_3N_2Cl$: N, 12.26%. Found: N, 12.25%.

m-Aminophenylacetic Methyl Ester Hydrochloride.—56 g. *m*-aminophenylacetic acid² were treated with 500 cc. of dry methyl alcohol and saturated with dry hydrochloric acid gas. The resulting solution was concentrated to dryness *in vacuo*, taken up in a small quantity of dry methyl alcohol, and precipitated with dry ether. After standing in the ice box the salt was filtered off and washed with dry ether. The yield was 60 g. A portion was recrystallized by dissolving in cold absolute alcohol, bone-black, filtering, and adding dry ether. It separated as long, thin, glistening plates which dissolve readily in the cold in methyl or ethyl alcohol. When rapidly heated to 160° and then slowly the salt softens and melts at 167–70° with slow gas evolution. It is readily diazotized, giving a scarlet color with R-salt. An aqueous solution, treated with sodium carbonate, gave the free ester as an oil which did not solidify.

0.1955 g. subst.; 0.1385 g. AgCl.

Calc. for $C_9H_{11}O_2N.HCl$: Cl, 17.59%. Found: Cl, 17.53%.

m-Aminophenylacetamide, *m-H₂NC₆H₄CH₂CONH₂*.—55 g. of the above hydrochloride were dissolved in 200 cc. of cold, concentrated, aqueous ammonia and let stand, with occasional shaking, for 24 hours. The amide which had separated was filtered off, washed with a little water, and dried. The yield was 36 g. A portion was recrystallized from water, decolorizing with bone-black, and adding a drop of

¹ Cf. THIS JOURNAL, 39, 1439 (1917).

² *Ibid.*, 39, 1437 (1917).

aqueous ammonia to the filtered solution. The amide separates slowly as transparent prisms which are anhydrous. Recrystallized again from 95% alcohol it forms leaflets which melt at 164–6°(cor.) with preliminary softening. It is readily soluble in boiling water, less easily in boiling 95% alcohol and sparingly so in acetone or ethyl acetate. The amide is readily diazotized.

Kjeldahl: 0.1402 g. subst.; 18.65 cc. 0.1 *N* HCl.

Calc. for $C_8H_{10}ON_2$: N, 18.67%. Found: N, 18.63%.

m-Chloroacetylaminophenylacetamide.—10 g. of the crude amide were chloroacetylated in a mixture of 50 cc. acetic acid and 50 cc. of saturated sodium acetate solution. The acyl derivative separated from the reaction mixture on scratching. After recrystallization from 50% alcohol the yield was 6 g. The substance forms prismatic needles which melt with slight preliminary softening at 187–8° to a pale brown liquid. The compound is readily soluble in boiling acetic acid, less easily in boiling water or 50% alcohol, and almost insoluble in chloroform.

Kjeldahl: 0.1451 g. subst.; 12.9 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{11}O_2N_2Cl$: N, 12.37%. Found: N, 12.45%

p-Aminophenylacetamide.¹

p-Chloroacetylaminophenylacetamide.¹

(3) Derivatives of the Aminophenoxyacetamides.

In a first attempt to prepare *o*-chloroacetylaminophenoxyacetamide, $o\text{-ClCH}_2\text{CONHC}_6\text{H}_4\text{OCH}_2\text{CONH}_2$, *o*-nitrophenoxyacetamide was reduced by the ferrous sulfate and ammonia method² with the expectation of obtaining *o*-aminophenoxyacetamide. The only produde isolated, however, was *o*-aminophenoxyacetic anhydride,³ which was formed by the elimination of ammonia from the intermediate *o*-aminophenoxyacetamide. The desired chloroacetyl compound was finally obtained by the series of reactions outlined below, a description of *o*-nitrophenoxyacetamide being given first.

¹ THIS JOURNAL, 39, 1444 (1917).

² *Ibid.*, 39, 1435 (1917).

³ *Ibid.*, 39, 2190 (1917).

o-Nitrophenoxyacetamide, $o\text{-O}_2\text{NC}_6\text{H}_4\text{OCH}_2\text{CONH}_2$.—47 g. *o*-nitrophenoxyacetic acid¹ were dissolved in 300 cc. of dry methyl alcohol and saturated with hydrochloric acid gas. The resulting methyl ester was dissolved in ether and the solution shaken with concentrated aqueous ammonia. The amide rapidly crystallized out and was filtered off and washed with water. The yield was 18 g. Recrystallized from water it forms long, glistening needles which melt at 194.5–5.5° (cor.). The amide is only sparingly soluble in hot water, 95% alcohol, or acetone. When boiled with dilute sodium hydroxide it is easily hydrolyzed.

Kjeldahl: 0.2180 g. subst.; 22.30 cc. 0.1 N HCl.

Calc. for $\text{C}_8\text{H}_8\text{O}_4\text{N}_2$: N, 14.29%. Found: N, 14.33%.

o-Chloroacetylaminophenoxyacetic Acid, $o\text{-ClCH}_2\text{CONHC}_6\text{H}_4\text{OCH}_2\text{CO}_2\text{H}$.—6 g. potassium *o*-aminophenoxyacetate were dissolved in 120 cc. 5% aqueous sodium hydroxide solution, turbined, and treated, drop by drop, with 4.5 cc. chloroacetyl chloride. At the end the solution was acidified with acetic acid, when it remained clear, showing the absence of unchanged *o*-aminophenoxyacetic anhydride (the free *o*-aminophenoxyacetic acid does not exist under ordinary conditions). On acidifying to Congo red with hydrochloric acid the *o*-chloroacetylaminophenoxyacetic acid separated. The yield was 5 g. It crystallizes from 50% acetic acid with 2 molecules of water of crystallization which are lost on heating *in vacuo*, finally at 110°. The air-dried substance melts partially and effervesces when rapidly heated to about 100°, while the anhydrous compound melts at 144.5–5.5° (cor.) with slight preliminary softening. The acid dissolves readily in acetone, less easily in chloroform, and very difficultly in benzene.

Air dry: 0.9989 g. subst.; 0.1350 g. loss. H_2O , 13.52%. Calc. for 2 H_2O : H_2O , 12.88%.

Anhydrous: Kjeldahl: 0.3088 g. subst.; 12.70 cc. 0.1 N HCl.

Calc. for $\text{C}_{10}\text{H}_{10}\text{O}_4\text{NCl}$: N, 5.75%. Found: N, 5.76%.

o-Chloroacetylaminophenoxyacetyl Chloride, $\text{ClCH}_2\text{CONHC}_6\text{H}_4\text{OCH}_2\text{COCl}$.—23 g. phosphorus pentachloride were added to a suspension of 23 g. *o*-chloroacetylaminophenoxyacetic acid in 50 cc. benzene. As the reaction proceeded hydrochloric acid was evolved and the acid

¹ THIS JOURNAL, 39, 2191 (1917).

went into solution. At the end ligroin was added until the precipitation of the chloride was complete. The product was filtered off and washed with ligroin. The yield was 19 g. Recrystallized from ligroin the chloride forms colorless plates which melt at $52-9^{\circ}$ with preliminary softening and dissolve readily in the usual organic solvents with the exception of ligroin.

Kjeldahl: 0.2025 g. subst.; 7.80 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_9O_2NCl_2$: N, 5.35%. Found: N, 5.39%.

o-Chloroacetylaminophenoxyacetamide.—18 g. of the chloride were dissolved in chloroform, chilled, and treated with concentrated aqueous ammonia. The amide, which separated at once, was more completely thrown out by the addition of petroleum ether and was filtered off and washed with water. Recrystallized from water it forms needles which melt constantly at $158-61^{\circ}$ with preliminary softening. The substance is sparingly soluble in cold water or 95% alcohol, readily in hot, and also dissolves in acetone or hot chloroform. It gives a strong Beilstein test.

0.1485 g. subst.; 14.6 cc. N, 765 mm., 19.0° .

Calc. for $C_{10}H_{11}O_2N_2Cl$: N, 11.55%. Found: N, 11.57%.

m-Aminophenoxyacetamide.—*m*-Aminophenoxyacetic ethyl ester hydrochloride¹ was dissolved in a little warm water, cooled, and shaken with concentrated aqueous ammonia. The oily ester gradually dissolved on shaking. After standing overnight the solution was evaporated to small bulk and cooled, whereupon the amide separated. This was filtered off, washed with a little water, and recrystallized, with bone-blackening, first from a little water, then from a small amount of 95% alcohol. It forms delicate, cream-colored needles which soften above 118° , melt partially at about 119° , and are completely fused at $123.5-24^{\circ}$ (cor.). The amide is soluble in water, alcohol, methyl alcohol, or acetone, and almost insoluble in benzene. It is readily diazotized.

Kjeldahl: 0.1782 g. subst.; 21.55 cc. 0.1 *N* HCl.

Calc. for $C_8H_{10}O_2N_2$: N, 16.87%. Found: N, 16.94%.

¹ THIS JOURNAL, 39, 2192 (1917).

m-Chloroacetylaminophenoxyacetamide.—The amide was chloroacetylated in a mixture of 5 parts of acetic acid and 5 parts of saturated sodium acetate solution. The chloroacetyl derivative separated from the reaction mixture after a few moments. Recrystallized from acetic acid, in which it is less sparingly soluble at the boiling point than in the other usual solvents, it forms radiating masses of microscopic leaflets. Rapidly heated to 230°, then slowly, it melts at 235–8° to a clear liquid which soon darkens and decomposes.

Kjeldahl: 0.1637 g. subst.; 13.25 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{11}O_2N_2Cl$: N, 11.55%. Found: N, 11.33%.

p-Aminophenoxyacetamide.—40 g. *p*-aminophenoxyacetic methyl ester hydrochloride¹ were added to 80 cc. of concentrated aqueous ammonia, with cooling. The free ester separated and gradually went into solution on shaking. The amide separated on scratching, and after standing for several hours was filtered off, washed with a little concentrated aqueous ammonia, and dried. The yield was 28 g. The amide is soluble in cold water, from which it is thrown out by alkalis. It separates from hot, concentrated aqueous solutions as glistening, brownish platelets. It is sparingly soluble in cold ethyl acetate, separating from the hot solution as cream-colored prisms which soften at 125° and melt at 127.5–8.5°. It is difficultly soluble in benzene or chloroform, readily in acetone. An aqueous solution gives a slowly developing rose color with ferric chloride and is readily diazotized, yielding a scarlet dye with R-salt.

Kjeldahl: 0.1972 g. subst.; 23.60 cc. 0.1 *N* HCl.

Calc. for $C_8H_{10}O_2N_2$: N, 16.87%. Found: N, 16.77%.

p-Chloroacetylaminophenoxyacetamide.—5 g. of the amide were chloroacetylated as in previous examples. The reaction mixture set to a thick mass. The yield of chloroacetyl derivative was 6.5 g. A portion was recrystallized first from 50% alcohol, then from water, forming practically colorless needles which melt at 195–6.5° (cor.) with slight preliminary softening. The compound is sparingly soluble

¹ THIS JOURNAL, 39, 2196 (1917).

in absolute alcohol or chloroform, and almost insoluble in benzene or cold water.

Kjeldahl: 0.1485 g. subst.; 12.25 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{11}O_2N_2Cl$: N, 11.55%. Found: N, 11.56%.

Hexamethylenetetraminium Salt of p-Chloroacetylaminophenoxyacetamide.—4.5 g. of the chloroacetyl derivative and 2.3 g. hexamethylenetetramine were boiled in 250 cc. acetone for 5 hours. The suspension of chloroacetyl compound gradually changed into a heavy precipitate consisting of prisms and plates. The product was filtered off and washed with dry acetone. The yield was 4.6 g. The salt is soluble in cold water, but very sparingly so in boiling acetone, absolute alcohol, or chloroform.

0.1811 g. subst.; 0.0664 g. AgCl.

Calc. for $C_{16}H_{23}O_3N_6Cl$: Cl, 9.27%. Found: Cl, 9.07%.

p-Nitrophenoxyacetyl Chloride.—Equimolecular amounts of *p*-nitrophenoxyacetic acid¹ and phosphorus pentachloride were warmed on the steam bath until evolution of hydrochloric acid had ceased and a homogeneous liquid was obtained. Ligroin was then added, precipitating the *p*-nitrophenoxyacetyl chloride in practically quantitative yield. A portion was recrystallized from benzene, forming transparent, hexagonal tablets which melt at 86–7° (cor.) with slight preliminary softening. The chloride is soluble in ether or benzene and reacts slowly in the cold with water or absolute alcohol.

0.2532 g. subst., boiled with aq. NH_3 ; 0.1679 g. AgCl.

Calc. for $C_8H_6O_4N_2Cl$: Cl, 16.45%. Found: Cl, 16.40%.

p-Nitrophenoxyacetmethylamide, $p-O_2NC_6H_4OCH_2CONHCH_3$.—22 g. *p*-nitrophenoxyacetyl chloride in dry chloroform were poured into a well chilled solution of an equimolecular amount (8 g.) of methylamine hydrochloride in 100 cc. of 10% aqueous sodium hydroxide. On shaking, the amide crystallized out at once, and was filtered off and washed with water. The yield was 19 g. The amide dissolves in boiling water with a pale yellow color and separates from the cooled solution in brilliant needles. Recrystallized again from toluene it melts at

¹ Cf. THIS JOURNAL, 39, 1437 (1917).

165.6° (cor.) with preliminary softening. It is rather difficultly soluble in hot absolute alcohol or toluene, more easily in hot water.

Kjeldahl: 0.1775 g. subst.; 16.80 cc. 0.1 *N* HCl.

Calc. for $C_9H_{10}O_4N_2$: N, 13.33%. Found: N, 13.26%.

p-Aminophenoxyacetmethylamide.—5 g. of the nitroamide were added in small portions to a solution of 25 g. stannous chloride in 50 cc. of concentrated hydrochloric acid immersed in a freezing mixture. The resulting crystalline precipitate was filtered off and dissolved in an excess of well-chilled 10% sodium hydroxide solution. The aminoamide was extracted from the solution by shaking out with ethyl acetate. This was dried, concentrated to small bulk, and the amide precipitated by the addition of petroleum ether. The yield was 2.7 g. Recrystallized twice from toluene, it forms glistening, cream-colored needles which melt at 109.5–11° (cor.) with slight preliminary softening. The amide is easily soluble in water, alcohol, or acetone, difficultly in cold toluene. It is readily diazotized. An aqueous solution gives a slowly developing violet rose color with ferric chloride.

Kjeldahl: 0.1539 g. subst.; 17.05 cc. 0.1 *N* HCl.

Calc. for $C_9H_{12}O_2N_2$: N, 15.56%. Found: N, 15.52%.

2-Methyl-4-aminophenoxyacetamide.—5 g. of 2-methyl-4-aminophenoxyacetic methyl ester hydrochloride¹ were treated with an excess of concentrated aqueous ammonia. The lumpy mass was ground up in a mortar and allowed to stand for several hours, setting to a solid cake. This was disintegrated, filtered, and washed with a little water. Recrystallized first from water, then from 95% alcohol, the amide forms long, flat, glistening needles which soften at 155° and melt at 155.5–56° (cor.). It is sparingly soluble in cold water or 95% alcohol, readily on boiling, and dissolves also in acetone or methyl alcohol. When diazotized, it yields a deep red color with R-salt. An aqueous solution gives a brown coloration with ferric chloride, changing through purple to violet.

Kjeldahl: 0.1602 g. subst.; 17.90 cc. 0.1 *N* HCl.

Calc. for $C_9H_{12}O_2N_2$: N, 15.56%. Found: N, 15.65%.

¹ THIS JOURNAL, 39, 2199 (1917).

3-Methyl-4-aminophenoxyacetamide.—2 g. 3-methyl-4-aminophenoxyacetic ethyl ester hydrochloride¹ were treated with an excess of concentrated aqueous ammonia and allowed to stand two days, with occasional stirring. The oily ester was gradually converted into the crystalline amide. This was filtered off and recrystallized twice from water, forming faintly pinkish, radiating masses of long, fine hairs which melt at 136–7° with slight preliminary softening and slight gas evolution. The amide is readily soluble in 85% alcohol or acetone, less easily in water or chloroform, and sparingly in benzene. It is easily diazotized, giving a red color with R-salt, and, in aqueous solution, yields a brown color with ferric chloride, rapidly changing to purple.

Kjeldahl: 0.1502 g. subst.; 16.60 cc. 0.1 N HCl.

Calc. for $C_9H_{12}O_2N_2$: N, 15.56%. Found: N, 15.48%.

2,5-Dimethyl-4-aminophenoxyacetamide.—3 g. 2,5-dimethyl-4-aminophenoxyacetic methyl ester hydrochloride² were suspended in alcohol and an excess of concentrated aqueous ammonia added. The amide separated from the clear solution on standing overnight. The mixture was concentrated to remove the alcohol and the amide then filtered off and recrystallized first from water, then from 95% alcohol. It forms delicate, silky needles which soften above 151.5° and melt at 153–4° (cor.). It is sparingly soluble in the cold in water, chloroform, or benzene, readily on warming, and is more soluble in the cold in 95% alcohol or acetone. It is readily diazotized, giving a carmine color with R-salt, and in aqueous solution yields a slowly developing, deep violet color with ferric chloride.

Kjeldahl: 0.1551 g. subst.; 15.85 cc. 0.1 N HCl.

Calc. for $C_{10}H_{14}O_2N_2$: N, 14.43%. Found: N, 14.32%.

2-Methyl-4-amino-5-isopropylphenoxyacetamide.—2 g. 2-methyl-4-amino-5-isopropylphenoxyacetic methyl ester hydrochloride³ were suspended in alcohol and treated with an excess of concentrated aqueous ammonia. After 2 days the clear solution was concentrated on the water bath and cooled after adding a few drops of ammonia. The

¹ THIS JOURNAL, 39, 2201 (1917).

² *Ibid.*, 39, 2205 (1917).

³ *Ibid.*, 39, 2206 (1917).

amide which separated was filtered off, dried, and recrystallized by dissolving in hot benzene, decolorizing with bone-black, adding ligroin until the turbidity first formed just dissolved, and seeding. 1.2 g. of the amide separated slowly in the form of aggregates of minute prisms and plates. Recrystallized again from water, from which it separates as slightly pinkish rhombs, then from toluene, the amide forms aggregates of diamond-shaped plates which melt at $108-9^{\circ}$ (cor.) and are soluble at room temperature in water and the usual organic solvents except ligroin. A diazotized solution of the amide gives a deep red color with R-salt, while an aqueous solution gives a slowly developing violet-blue with ferric chloride. A dilute acetic acid solution gives a transitory blue color with sodium nitrite.

Kjeldahl: 0.1532 g. subst.; 13.75 cc. 0.1 *N* HCl.

Calc. for $C_{12}H_{18}O_2N_2$: N, 12.62%. Found: N, 12.57%.

3-Methyl-4-amino-6-isopropylphenoxyacetamide.—3 g. 3-methyl-4-amino-6-isopropylphenoxyacetic methyl ester hydrochloride¹ were taken up in alcohol and treated with an excess of concentrated aqueous ammonia, enough alcohol being present to prevent separation of the oily ester. After standing overnight the solution was evaporated to small bulk on the water bath, treated with a few drops of ammonia, cooled, and stirred, whereupon the amide crystallized. It was filtered off and recrystallized, first from water, using bone-black, then from toluene, forming pointed leaflets which soften at 124.5° and melt at $125-5.5^{\circ}$ (cor.). It is sparingly soluble in cold water, more readily in hot, melting down before dissolving, and separating on cooling as pinkish needles. It is readily soluble in the cold in acetone, alcohol, or chloroform; difficultly in cold benzene or toluene and readily on warming. An aqueous suspension gives a gray color with ferric chloride, changing through purple to violet-blue. When diazotized the amide gives a carmine color with R-salt.

Kjeldahl: 0.1454 g. subst.; 12.95 cc. 0.1 *N* HCl.

Calc. for $C_{12}H_{18}O_2N_2$: N, 12.62%. Found: N, 12.48%.

2-Bromo-4-aminophenoxyacetamide.—3 g. 2-bromo-4-aminophenoxyacetic methyl ester hydrochloride² were taken up in alcohol and treated

¹ THIS JOURNAL, 39, 2207 (1917).

² *Ibid.*, 39, 2209 (1917).

with an excess of concentrated aqueous ammonia. After two days the clear solution was concentrated on the water bath and treated with a few drops of ammonia. The amide separated and was filtered off and recrystallized first from water, using bone-black, then from a small volume of 95% alcohol. It forms radiating masses of long, flat, cream-colored needles which melt at 159–60° (corr.) with slight preliminary softening. It is soluble in acetone or boiling water, more readily in boiling 95% alcohol, and sparingly in the last two in the cold. The amide is readily diazotized and, in aqueous suspension, gives a slowly developing wine-red color with ferric chloride.

Kjeldahl: 0.2649 g. subst.; 21.70 cc. 0.1 *N* HCl.

Calc. for $C_8H_9O_2N_2Br$: N, 11.43%. Found: N, 11.47%.

4-Amino-6-methoxyphenoxyacetamide.—4 g. 4-amino-6-methoxyphenoxyacetic ethyl ester hydrochloride¹ were decomposed with aqueous ammonia. On standing the oily ester gradually changed to the crystalline amide. This was filtered from the deep blue solution and recrystallized first from water, with bone-black, then from 95% alcohol. It forms almost colorless, glistening needles which melt at 177.5–8.5° (cor.) with preliminary softening. It is sparingly soluble in cold water or 95% alcohol, readily on heating, and also dissolves with difficulty in acetone, the crystalline form changing on heating with this solvent. When diazotized it gives a purplish red color with R-salt, while in aqueous solution it gives a brown color with ferric chloride, rapidly changing through purple to brown-lilac.

Kjeldahl: 0.1560 g. subst.; 16.00 cc. 0.1 *N* HCl.

Calc. for $C_9H_{12}O_3N_2$: N, 14.29%. Found: N, 14.37%.

4-Amino-6-carbethoxyphenoxyacetamide.—1.8 g. 4-amino-6-carbethoxyphenoxyacetic ethyl ester hydrochloride² were treated with an excess of concentrated aqueous ammonia. The oily ester gradually changed to the crystalline amide. After standing overnight the amide was filtered off and recrystallized twice from 95% alcohol, forming minute leaflets which melt at 135–6° (cor.) with preliminary softening. It is difficultly soluble in cold water, readily on boiling, and is quite

¹ THIS JOURNAL, 39, 2212 (1917).

² *Ibid.*, 39, 2214 (1917).

soluble in alcohol at room temperature, less easily at 0° . On adding sodium hydroxide to an aqueous solution the amide is salted out, but on boiling it redissolves, ammonia is evolved, and the solution gives the iodoform test, thus demonstrating both the amide and ester functions.

Kjeldahl: 0.1917 g. subst.; 15.65 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{14}O_4N_2$: N, 11.76%. Found: N, 11.43%.

4-Amino-6-acetophenoxyacetamide.—3 g. 4-amino-6-acetophenoxyacetic methyl ester hydrochloride¹ were treated with alcohol and concentrated aqueous ammonia, using sufficient alcohol to keep the ester from separating. After several days the solution was concentrated on the water bath, adding a few drops of ammonia at the end. The amide, which separated on cooling, was recrystallized, first from a small volume of water, using bone-black, then from a small amount of 95% alcohol. It forms brownish yellow leaflets, many of which are diamond-shaped. The amide melts at $138-9^{\circ}$ with preliminary softening and slight gas evolution. It is easily soluble in boiling water or 95% alcohol, sparingly in the cold, and dissolves in dilute hydrochloric acid without color. It is soluble in acetone; difficultly in benzene. An aqueous solution gives a slowly developing, deep brown color with ferric chloride.

Kjeldahl: 0.2539 g. subst.; 24.20 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{12}O_3N_2$: N, 13.47%. Found: N, 13.36%.

(4) *Derivatives of Aminobenzenesulfonamides.*

m-Aminobenzenesulfonamide is perhaps most easily accessible through the reduction of the nitro compound. As we were in the possession of a considerable quantity of metanilic acid we decided to attempt the preparation with this as starting material. The reaction was carried out in the following steps: metanilic acid \rightarrow sodium *m*-acetaminobenzenesulfonate \rightarrow *m*-acetaminobenzenesulfone chloride \rightarrow *m*-acetaminobenzenesulfonamide \rightarrow *m*-aminobenzenesulfonamide. The only new substance isolated in a state of purity was the *m*-acetaminobenzenesulfonamide.

m-Acetaminobenzenesulfonamide, $m\text{-CH}_3\text{CONHC}_6\text{H}_4\text{SO}_2\text{NH}_2$.—129 g. metanilic acid were acetylated by dissolving in 750 cc. of normal so-

¹ THIS JOURNAL, 39, 2215 (1917).

dium hydroxide solution (1 mol), adding 90 cc. acetic anhydride (1.2 mols), and shaking. The resulting solution was concentrated to dryness *in vacuo* and the residue of sodium *m*-acetaminobenzenesulfonate taken up in acetone, filtered off, and dried at 120°. The yield of crude salt was 155 g. 50 g. of this were ground up in a mortar with 50 g. phosphorus pentachloride, the mass liquefying, evolving hydrochloric acid, and finally hardening to some extent. Ice-water was then added, yielding two layers, of which the upper was decanted and the lower layer of crude chloride washed twice more with ice-water by decantation. The product was then ground up with 50 cc. of aqueous ammonia (d. 0.94), causing gradual crystallization of the amide. This was filtered off and washed with a little water. The yield was 11.2 g. Recrystallized first from 50% alcohol, then from acetic acid, the amide forms minute rhombs which melt constantly at 216–9° with slight preliminary softening. When pure the amide is soluble in acetone or hot acetic acid and sparingly so in the other usual solvents, while the crude substance is considerably more soluble. When hydrolyzed with 1:1 hydrochloric acid it yields *m*-aminobenzenesulfonamide in about 75% of the theory and corresponding in its properties with those recorded in the literature.

Kjeldahl: 0.1495 g. subst.; 13.85 cc. 0.1 *N* HCl.

Calc. for $C_8H_{10}O_3N_2S$: N, 13.08%. Found: N, 12.98%.

m-Chloroacetylaminobenzenesulfonamide.—18.8 g. *m*-aminobenzene-sulfonamide were brought into solution in a mixture of 95 cc. acetic acid and 95 cc. saturated sodium acetate solution by warming gently. The solution was cooled to 0° and treated, drop by drop, with shaking and cooling, with 11.3 cc. chloroacetyl chloride. The chloroacetyl derivative separated on scratching and was filtered off and combined with an additional amount obtained by concentrating the filtrate *in vacuo*. After recrystallization from water the yield was 24.5 g. Recrystallized, with bone-blackening, from 95% alcohol the compound forms aggregates of minute needles which soften slightly when heated, melt incompletely at 153–5°, and form a clear melt at about 165°. It dissolves readily in boiling water, sparingly in the cold, is almost insoluble in chloroform or benzene, and dissolves in acetone or 95% alcohol at room temperature.

Kjeldahl: 0.1540 g. subst.; 12.25 cc. 0.1 *N* HCl.

Calc. for $C_8H_8O_3N_2ClS$: N, 11.27%. Found: N, 11.14%.

p-Chloroacetylaminobenzenesulfonamide. — *p*-Aminobenzenesulfonamide was prepared essentially as directed by Gelmo,¹ the only new feature being the acetylation of sodium sulfanilate as given above in the case of the *meta* isomer. 10 g. of the amide were warmed with 50 cc. 50% acetic acid, 50 cc. saturated sodium acetate solution, and 50 cc. acetic acid until dissolved, cooled in ice-water, and treated in the usual way with 6 cc. chloroacetyl chloride. The chloroacetyl derivative separated almost immediately and was filtered off and washed well with 50% acetic acid and water. Recrystallized from 95% alcohol it forms needles which melt with slight preliminary softening at 215–7° to a clear liquid which soon darkens. The substance is soluble in hot water or 95% alcohol, cold ethyl acetate or acetone, and difficultly so in chloroform or benzene. It also dissolves in sodium hydroxide solution.

Kjeldahl: 0.1433 g. subst.; 11.55 cc. 0.1 *N* HCl.

Carius: 0.1820 g. subst.; 0.1037 g. AgCl, 0.1733 g. BaSO₄.

Calc. for $C_8H_8O_3N_2ClS$: N, 11.27%; Cl, 14.26%; S, 12.90%.

Found: N, 11.29%; Cl, 14.09%; S, 13.08%.

(B) Ureides Containing the Aromatic Nucleus.

(1) Ureides of Substituted Benzoic Acids.

o-Aminobenzoylurea.²

o-Chloroacetylaminobenzoylurea, $o\text{-ClCH}_2\text{CONHC}_6\text{H}_4\text{CONHCONH}_2$. — 10 g. *o*-aminobenzoylurea were dissolved by warming with 300 cc. 50% acetic acid, 25 cc. saturated sodium acetate solution, and 25 cc. acetic acid. The solution was rapidly cooled until lukewarm and treated with 7 cc. chloroacetyl chloride. The chloroacetyl derivative slowly separated. After cooling and letting stand the product was filtered off, washed with 2.5% hydrochloric acid containing a little alcohol to remove unchanged amino compound, and finally washed with water and dried. The yield was 6.2 g. Recrystallized from 50% alcohol it forms long, glistening needles which are difficultly

¹ Gelmo, *J. prakt. Chem.*, [2] 77, 369 (1907).

² THIS JOURNAL, 39, 1438 (1917).

soluble in water, dilute or 95% alcohol, and acetone at their boiling points. When rapidly heated to 220°, then slowly, it softens slightly and melts with effervescence at 222–3°, then darkens and decomposes. On boiling in aqueous solution with sodium hydroxide, ammonia is evolved and the resulting solution contains chlorine ion.

0.1202 g. subst.; 17.2 cc. N, 760 mm., 22.5°.

Calc. for $C_{10}H_{10}O_3N_3Cl$: N, 16.44%. Found: N, 16.53%.

m-Aminobenzoylurea.¹

m-Chloroacetylaminobenzoylurea.—12 g. *m*-aminobenzoylurea were dissolved in a mixture of 150 cc. 50% acetic acid and 150 cc. acetone. 20 g. sodium acetate were then added, followed by 8 cc. chloroacetyl chloride, added drop by drop, with vigorous shaking. The chloroacetyl derivative separated at once and was filtered off after diluting the mixture with water. The yield was 14 g. Recrystallized from water, it forms delicate needles which are difficultly soluble in the usual solvents. When rapidly heated to 250°, then slowly, it decomposes at 252–3°.

0.1122 g. subst.; 16.0 cc. N, 758 mm., 20.5°.

Calc. for $C_{10}H_{10}O_3N_3Cl$: N, 16.44%. Found: N, 16.55%.

p-Nitrobenzoylurea, $p-O_2NC_6H_4CONHCONH_2$.—40 g. *p*-nitrobenzoyl chloride and 30 g. urea were boiled in 100 cc. benzene on the water bath for several hours. After cooling the mixture was treated with water, filtered, washed with water and acetone, and dried. The yield was 38 g. Recrystallized from acetic acid, it forms aggregates of microscopic prisms which are less difficultly soluble in acetic acid than in the other usual solvents. When rapidly heated to 240°, then slowly, it melts at 243–5° with gas evolution and slight preliminary softening.

0.0902 g. subst.; 15.8 cc. N, 767 mm., 22.0°.

Calc. for $C_8H_7O_4N_3$: N, 20.10%. Found: N, 20.46%.

p-Aminobenzoylurea.—35 g. of the finely powdered nitrobenzoylurea were heated on the water bath in a mixture of 400 cc. ammonium sulfide solution and 100 cc. alcohol, with frequent shaking. The character of the precipitate suddenly changed, indicating that reduction had

² THIS JOURNAL, 39, 1439 (1917).

taken place. After one hour's heating the aminobenzoylurea was filtered off and washed well with water. The yield of crude product was 30 g. For analysis a portion was ground up with dilute sulfuric acid and treated with water until most of the material had dissolved. The solution was filtered and the free base precipitated with ammonia. The urea was filtered off and washed successively with water, alcohol, and benzene. It is very difficultly soluble in the usual solvents, including acids, and separates from boiling water as minute, colorless plates. A suspension in dilute hydrochloric acid yields a soluble diazonium salt with sodium nitrite, coupling with R-salt to give a scarlet color. The urea decomposes partly at about 240° but does not melt below 285° .

Kjeldahl: 0.1113 g. subst.; 18.50 cc. 0.1 N HCl.

Calc. for $C_8H_9O_2N_3$: N, 23.46%. Found: N, 23.29%.

The nitrobenzoylurea may also be reduced by the ferrous sulfate and ammonia method,¹ adding an equal volume of alcohol to the mixture after reducing and again bringing to a boil before filtering. The yield by this method, however, was only about 40% of the theory.

p-Chloroacetylaminobenzoylurea.—Owing to the sparing solubility of *p*-aminobenzoylurea in dilute acetic acid the chloroacetylation is best accomplished by the procedure used by us for chloroacetylating the uramino group.²

10 g. chloroacetic acid were melted on the water bath and 3 g. crude *p*-aminobenzoylurea added to the melt. To the suspension so obtained 2.1 cc. chloroacetyl chloride were added, causing an immediate reaction marked by the evolution of hydrochloric acid gas and solidification of the mixture. An additional 5 g. chloroacetic acid were added to increase the fluidity of the mixture, the lumpy mass was disintegrated and the heating continued for a total of one-half hour. Water was then added and the product filtered off and washed well with water. The yield of crude product was 4.6 g. A portion was recrystallized from a large volume of hot water, forming minute needles and prisms which dissolve with less difficulty in boiling acetic acid than in the other usual solvents. When rapidly heated to 275° ,

¹ *Loc. cit.*

² THIS JOURNAL, 39, 1446 (1917).

then slowly, the urea decomposes at 275–80° with slight preliminary softening.


Kjeldahl: 0.1000 g. subst.; 11.65 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{10}O_3N_3Cl$: N, 16.44%. Found: N, 16.32%.

Salicylylurea (*o*-hydroxybenzoylurea), *o*- $HOC_6H_4CONHCONH_2$.—47 g. of finely powdered urea were added to a solution of 75 g. acetylsalicylyl chloride in 280 cc. benzene. The mixture was boiled under a reflux condenser for 6–8 hours and the supernatant liquid poured off. The residue was ground up in a mortar with water, filtered off, and thoroughly washed with water. The crude acetylsalicylyl urea so obtained was suspended in water, turbined, and carefully treated with sodium hydroxide in the cold so that the reaction of the mixture was kept just alkaline to phenolphthalein. When permanent alkalinity was reached the solution was filtered from a small amount of unchanged material and acidified with acetic acid. The pale yellow precipitate which formed was filtered off and recrystallized from 50% alcohol, giving the salicylylurea in a yield of 11.1 g. as pale yellow microcrystals which melt with effervescence at 184–6°. In 50% alcohol it gives a brown color with ferric chloride. The urea is very difficultly soluble in acetone or benzene, sparingly soluble in ethyl acetate, hot water, or methyl and ethyl alcohols in the cold, and soluble in hot methyl or ethylalcohol. It dissolves in dilute ammonia, yielding a solution which couples readily with diazotized sulfanilic acid.

0.1113 g. subst.; 15.0 cc. N, 761 mm., 21.5°.

Calc. for $C_8H_8O_3N_2$: N, 15.56%. Found: N, 15.64%.

m(?)-Chloroacetylaminomethylbenzoylurea (*m*-Carboxureidochloroacetylbenzylamine,  $CH_2NHCOCH_2Cl$).—16.4 g. benzoylurea were dis-

solved in 100 g. sulfuric acid and treated, with cooling, with 12.3 g. methylolchloroacetamide, the procedure being that of Einhorn¹ for introducing the $CH_2NHCOCH_2Cl$ group into aromatic compounds. After standing for 2 days the clear solution was poured onto ice and the resulting white precipitate filtered off and washed well with

¹ Einhorn, *et al.*, *Ann.*, **343**, 207 (1905); **361**, 113 (1908).

water. In order to remove all adherent sulfuric acid the product was ground up in a mortar with sodium acetate solution until neutral to Congo red. The yield was 20 g. Recrystallized from acetic acid it forms a microcrystalline powder which begins to darken above 190° , softens above 200° , and melts at about $225-8^{\circ}$ with decomposition. It is very difficultly soluble in the usual solvents with the exception of boiling acetic acid. On boiling with dilute sodium hydroxide ammonia is evolved and the solution contains chlorine ion. In the absence of definite proof it is held likely that the chloroacetylaminomethyl group is in the *meta* position in accordance with the usual directive influence of the carbonyl group.

Kjeldahl: 0.1469 g. subst.; 16.50 cc. 0.1 N HCl.

Calc. for $C_{11}H_{12}O_3N_3Cl$: N, 15.59%. Found: N, 15.73%.

(2) Derivatives of Phenylacetylurea.

Phenylchloroacetylurea, $C_6H_5CHClCONHCONH_2$.—30 g. phenylchloroacetylchloride,¹ 19 g. urea (2 mols), and 200 cc. benzene were heated on the water bath under a reflux condenser for about 5 hours, occasionally breaking up the solid mass which formed. The product was ground up in a mortar with water, filtered, washed well with water, and recrystallized from 95% alcohol. The yield was 23.8 g., 5.8 g. of this having been recovered from the alcoholic mother liquor on concentration. The urea forms delicate needles which melt with slight preliminary softening at 198° to a yellow liquid which evolved gas. It is sparingly soluble in cold methyl alcohol or acetone, readily on warming, and is very difficultly soluble at the boiling point in water, benzene, or chloroform.

Kjeldahl: 0.2303 g. subst.; 21.60 cc. 0.1 N HCl.

Calc. for $C_9H_9O_2N_2Cl$: N, 13.18%. Found: N, 13.13%.

p-Nitrophenylacetylurea, $p-O_2NC_6H_4CH_2CONHCONH_2$.—Crude *p*-nitrophenylacetyl chloride was obtained by treating 63 g. *p*-nitrophenylacetic acid with 75 g. phosphorus pentachloride in benzene solution and concentrating *in vacuo*. To a solution of the chloride in 180 cc. benzene 42 g. (2 mols) urea were added and the mixture heated for about 6 hours on the water bath, the lumpy mass being

¹ Staudinger and Bereza, *Ber.*, **44**, 536 (1911).

occasionally broken up with a glass rod. At the end the supernatant liquid was poured off and the solid ground up with water, adding sodium acetate solution until neutral to Congo red. The urea was then filtered off and washed well with water. The yield was 67 g. A small portion was recrystallized from water, from which it separates as delicate, hair-like needles which are difficultly soluble in boiling water or 95% alcohol. The compound softens and turns yellow above 225° and melts with effervescence to an orange-brown liquid at 250–2°.

Kjeldahl: 0.2375 g. subst.; 31.55 cc. 0.1 *N* HCl.

Calc. for $C_9H_9O_4N_3$: N, 18.83%. Found: N, 18.61%.

p-Aminophenylacetylurea.—55.5 g. *p*-nitrophenylacetylurea were added to a boiling solution of 470 g. ferrous sulfate in 1600 cc. water. The mixture was made alkaline with ammonia, shaking vigorously, and the boiling continued for about 10 minutes. 1600 cc. alcohol were then added and the mixture digested for about three-quarters of an hour on the water bath. On filtering the hot solution and concentrating the filtrate to small bulk the aminourea was obtained in a yield of 35 g. Recrystallized from 50% alcohol, adding a few drops of ammonia to the solution before cooling, the urea forms almost colorless, felted needles which are soluble in boiling water, more easily in hot methyl alcohol, and sparingly so in ethyl alcohol. It melts at 198–9° with gas evolution. The diazotized compound couples with R-salt to form a red dye which changes to purplish brown with an excess of sodium carbonate.

Kjeldahl: 0.1014 g. subst.; 15.7 cc. 0.1 *N* HCl.

Calc. for $C_9H_{11}O_2N_3$: N, 21.76%. Found: N, 21.69%.

p-Chloroacetylaminophenylacetylurea.—20 g. sodium acetate were added to a solution of 12 g. *p*-aminophenylacetylurea in 120 cc. 50% acetic acid. 8 cc. chloroacetyl chloride were then added to the solution, with vigorous shaking, causing immediate precipitation of the chloroacetyl derivative. After diluting with water the substance was filtered off and washed well with water. The yield was 16 g. Recrystallized from 95% alcohol it forms delicate needles which dissolve sparingly in boiling water, more easily in boiling alcohol, and readily

in boiling acetic acid. When rapidly heated to 235° , then slowly, the urea melts at 241° with gas evolution and preliminary softening and darkening.

Kjeldahl: 0.1108 g. subst.; 12.45 cc. 0.1 N HCl.

Calc. for $C_{11}H_{12}O_8N_3Cl$: N, 15.59%. Found: N, 15.74%.

(3) *Derivatives of Aminophenoxyacetylureas.*

o-Nitrophenoxyacetyl Chloride, $o-O_2NC_6H_4OCH_2COCl$.—45 g. *o*-nitrophenoxyacetic acid, 54 g. phosphorus pentachloride, and 50 cc. toluene were heated on the water bath until evolution of hydrochloric acid ceased. The solution was then concentrated *in vacuo* and the residue dissolved in an equal volume of toluene. On adding petroleum ether until just turbid and scratching the chloride separated in large plates. More ligroin was added to complete the deposition of the chloride, which was then filtered off, washed with ligroin, and dried. The yield was 45 g. A portion was recrystallized from hot ligroin (b. $80-90^{\circ}$), adding a little ligroin to the solution as it cooled, in order to prevent too early deposition of the chloride as an oil. As obtained in this way it forms long, silky, faintly yellow needles which melt at $41-2^{\circ}$ (cor.) with slight preliminary softening. It dissolves readily in the usual organic solvents with the exception of ligroin.

0.1847 g. subst., boiled with aq. NH_3 ; 0.1204 g. AgCl.

Calc. for $C_8H_5O_4NCl$: Cl, 16.45%. Found: Cl, 16.13%.

o-Nitrophenoxyacetylurea.—40 g. of the chloride were dissolved in 100 cc. benzene and heated on the water bath for 5 hours with 25 g. of well powdered urea. The mixture was then ground up with water, filtered off, and thoroughly washed with water. The yield of crude product was 44 g. A portion was recrystallized from 50% alcohol, forming cream-colored, irregular prisms and long needles which change into the prisms on letting the mixture stand overnight. A little ammonia was added before filtering off, in order to hold back any saponified material. When rapidly heated to 180° , then slowly, the urea softens and finally melts at $186-8^{\circ}$ with slow gas evolution. It dissolves in the cold in acetone and in boiling water or alcohol.

0.1276 g. subst.; 19.8 cc. N, 756 mm., 25.0° .

Calc. for $C_9H_9O_6N_3$: N, 17.57%. Found: N, 17.68%.

An attempt to reduce the nitrophenoxyacetylurea resulted only in the isolation of *o*-aminophenoxyacetic anhydride. This is the more easily understood by the ease with which the nitro urea undergoes saponification, short boiling with dilute ammonia sufficing to produce a deep orange color in the originally almost colorless solution.

p-Nitrophenoxyacetylurea.—This was obtained from *p*-nitrophenoxyacetyl chloride (see p. 252) and urea as in previous examples. Recrystallized from acetic acid it forms minute crystals which are less difficultly soluble in boiling acetic acid than in the other usual solvents. When quickly heated to 245°, then slowly, it softens slightly and then melts at 250° with gas evolution to a yellow liquid which soon darkens.

Kjeldahl: 0.2145 g. subst.; 26.70 cc. 0.1 *N* HCl.

Calc. for $C_9H_9O_5N_3$: N, 17.57%. Found: N, 17.43%.

p-Aminophenoxyacetylurea.—50 g. of the well powdered nitrourea were added to a boiling solution of 450 g. ferrous sulfate in 1100 cc. water. The mixture was made alkaline with ammonia, shaking vigorously, diluted with 1100 cc. alcohol, again brought to a boil, and filtered hot. The amino compound separated from the filtrate on cooling in a yield of 20 g. Recrystallized from 50% alcohol, adding a few drops of ammonia to hold back any acid present, the urea forms long needles which soften above 190°, melt with gas evolution at 198–9°, resolidify, and melt again with gas evolution above 240°. It is soluble in boiling water, difficultly in boiling acetone or absolute alcohol. An aqueous suspension gives a slowly developing reddish color with ferric chloride, while a diazotized solution couples with R-salt to give a deep red color.

0.1134 g. subst.; 19.65 cc. N, 763 mm., 23.0°.

Calc. for $C_9H_{11}O_3N_3$: N, 20.09%. Found: N, 20.06%.

p-Chloroacetylaminophenoxyacetylurea.—This substance was obtained by chloroacetylating the aminourea in 50% acetic acid solution as in numerous preceding examples. The chloroacetyl derivative was filtered from the diluted reaction mixture and washed with water. Recrystallized from acetic acid it forms aggregates of minute, flat needles which melt and decompose at 238–40° with preliminary soft-

ening. The urea is practically insoluble in the usual solvents with the exception of acetic acid.

0.1328 g. subst.; 17.3 cc. N, 756 mm., 26.0°.

Calc. for $C_{11}H_{12}O_4N_3Cl$: N, 14.72%. Found: N, 14.80%.

Owing to the poor yield obtained in the reduction of *p*-nitrophenoxyacetylurea a method was sought by which to prepare *p*-chloroacetylaminophenoxyacetylurea in better yield, reckoning from the starting material. This was accomplished by the following series of steps:

p-Chloroacetylaminophenoxyacetic Acid, *p*-ClCH₂CONHC₆H₄OCH₂CO₂H.—10 g. *p*-aminophenoxyacetic acid were dissolved in an excess of 10% sodium hydroxide solution and treated with 6 cc. chloroacetyl chloride, with shaking and chilling. On acidification with hydrochloric acid the chloroacetyl compound separated as a thick mass. This was filtered off, washed with water, and dried. The yield was 12 g. Recrystallized first from water, then from acetic acid, the substance forms practically colorless rosetts of delicate needles which contain one molecule of acetic acid of crystallization which is lost at 100° *in vacuo* over sulfuric acid. When the air-dry substance is boiled with water acetic acid is evolved. After freeing from acetic acid the compounds melts with preliminary softening, at 167–9° to a brown liquid which clears completely at 170°.

Air-dry: 1.0325 g. subst.; loss, 0.1965 g. HOAc, 19.03%.

Calc. for $C_{10}H_{10}O_4NCl \cdot CH_3CO_2H$: HOAc, 19.76%.

Anhydrous: Kjeldahl: 0.3242 g. subst.; 13.40 cc. 0.1 N HCl.

Calc. for $C_{10}H_{10}O_4NCl$: N, 5.75%. Found: N, 5.79%.

p-Chloroacetylaminophenoxyacetyl Chloride.—30 g. of the acid were suspended in 60 cc. of dry benzene and treated with 30 g. phosphorus pentachloride. A vigorous reaction occurred, but the substance did not dissolve. The mixture was finally warmed on the water bath until the evolution of hydrochloric acid ceased, after which it was cooled and filtered. The resulting chloride was washed with benzene and dried *in vacuo* over sulfuric acid, the yield of crude product being 34 g. Recrystallized from toluene it forms aborescent aggregates of platelets which melt at 147–52° with gas evolution and subsequent darkening. The chloride is soluble in dry acetone but dissolves only sparingly in dry chloroform or toluene.

Kjeldahl: 0.1951 g. subst.; 7.5 cc. 0.1 N HCl.

Calc. for $C_{10}H_9O_3NCl_2$: N, 5.35%. Found: N, 5.38%.

16 g. of the chloride were boiled in benzene with 7.5 g. urea for about 8 hours and worked up as in previous examples. A quantitative yield of *p*-chloroacetylaminophenoxyacetylurea was obtained in this manner, the product being identical in every way with that prepared by chloroacetylating *p*-aminophenoxyacetylurea.

p-Chloroacetylaminophenoxyacetic Methyl Ester.—A small portion of the chloride was boiled with dry methyl alcohol until dissolved and the solution then evaporated to small bulk. On diluting with hot water and cooling cautiously the ester was obtained crystalline. It was filtered off, washed with a little very dilute sodium carbonate solution to remove any acid that had been formed, and recrystallized from 85% alcohol, forming flat needles which melt at $170-3^\circ$ with preliminary softening and slight browning. It is soluble in acetone or hot alcohol and but sparingly in benzene or boiling water.

0.1585 g. subst.; 8.0 cc. N, 746 mm., 23.0° .Calc. for $C_{11}H_{12}O_4NCl$: N, 5.44%. Found: N, 5.71%.

(C) *Aromatic Ureas Containing an Amide Group.*

o-Uraminobenzamide, *o*- $H_2NCONHC_6H_4CONH_2$.—20 g. *o*-aminobenzamide¹ were dissolved in 200 cc. 50% acetic acid, chilled in ice-water, and treated with a solution of 12.5 g. potassium cyanate. On scratching, 17 g. of the urea separated as thick, cream-colored, hexagonal plates. On attempting to recrystallize the substance from boiling water, copious evolution of ammonia occurred, ring condensation taking place with formation of benzoyleneurea as long needles. (Calc. for $C_8H_6O_2N_2$: N, 17.28%. Found: N, 17.33%.) The crude, air-dried *o*-uraminobenzamide was therefore analyzed. When rapidly heated to 180° , then slowly, it melts, effervesces, and resolidifies at $184-5^\circ$ and does not remelt up to 280° .

0.1037 g. subst.; 21.0 cc. N, 770 mm., 22.0° .Calc. for $C_8H_6O_2N_2$: N, 23.46%. Found: N, 23.74%.

m-Uraminobenzamide.—This substance was first prepared by Menschutkin,² who does not give a melting point. 20 g. of the hy-

¹ *Loc. cit.*² Menschutkin, *Ann.*, 158, 96 (1871).

drate of *m*-aminobenzamide were treated as in the case of the *o*-isomer. The yield of urea was 20 g. Recrystallized from water it forms flat needles. When rapidly heated to 230°, then slowly it melts with effervescence at about 235°, resolidifying and turning yellow on further heating, but not melting below 280°. If slowly heated it softens and evolves gas above 220°, but does not melt.

Kjeldahl: 0.1171 g. subst.; 19.60 cc. 0.1 *N* HCl.

Calc. for $C_8H_9O_2N_3$: N, 23.46%. Found: N, 23.45%.

m-Chloroacetyluraminobenzamide, *m*-ClCH₂CONHCONHC₆H₄-CONH₂.—10 g. *m*-uraminobenzamide were dissolved in 30 g. chloroacetic acid on the water bath, treated with 6 cc. chloroacetyl chloride, and heated one-half hour. The chloroacetyl derivative separated in a yield of 9 g. on pouring the melt into water. Recrystallized from water it forms aggregates of microscopic needles which decompose at 223–4° with preliminary darkening and softening. It is very sparingly soluble in hot water, acetone, or alcohol, and dissolves readily in hot acetic acid, difficultly in the cold.

Kjeldahl: 0.1000 g. subst.; 11.7 cc. 0.1 *N* HCl.

Calc. for $C_{10}H_{10}O_3N_3Cl$: N, 16.44%. Found: N, 16.40%.

p-Uraminobenzamide.—20 g. *p*-aminobenzamide were dissolved in 100 cc. of warm 50% acetic acid, quickly chilled, and treated with a concentrated aqueous solution of 13 g. potassium cyanate. The urea separated rapidly in a yield of 18 g. Recrystallized from water adding a few drops of ammonia to the warm solution, it forms aggregates of prisms which dissolve readily in boiling water, difficultly in boiling absolute alcohol or amyl alcohol, and are almost insoluble in acetone or benzene. When rapidly heated to 240° it melts with gas evolution at 240–2°, resolidifies, and gradually melts again with continued gas evolution as the temperature is raised; forming a clear, yellow liquid at 275°.

Kjeldahl: 0.1311 g. subst.; 21.75 cc. 0.1 *N* HCl.

Calc. for $C_8H_9O_2N_3$: N, 23.46%. Found: N, 23.24%.

p-Chloroacetyluraminobenzamide.—On adding 8.5 cc. chloroacetyl chloride to a partial solution of 15 g. *p*-uraminobenzamide in 45 g.

chloroacetic acid on the water bath a clear solution was obtained. In a few moments the mixture set to a solid mass of the chloroacetyl derivative. After heating 15 minutes longer it was diluted with water and the precipitate filtered off. The yield was 12 g. Recrystallized from 50% alcohol, it forms hexagonal plates and prisms which darken and soften above 210° and decompose at $236-7^{\circ}$. It is sparingly soluble in the usual neutral solvents, but dissolves in dilute aqueous sodium hydroxide.

Kjeldahl: 0.1455 g. subst.; 16.95 cc. 0.1 N HCl.

Calc. for $C_{10}H_{10}O_2N_3Cl$: N, 16.44%. Found: N, 16.33%.

m-Uraminophenylacetamide, *m*- $H_2NCONHC_6H_4CH_2CONH_2$.—15 g. *m*-aminophenylacetamide (p. 247) were dissolved in 100 cc. of normal hydrochloric acid and a few cc. of acetic acid added. The solution was then treated, with chilling, with 8.5 g. potassium cyanate dissolved in a little water. The urea separated on scratching, after which the solution was made acid to Congo red and the urea filtered off and washed with water. The yield was 15 g. Recrystallized, with bone-blackening, from water, it forms minute, practically colorless plates which are soluble in hot water, sparingly so in hot 95% alcohol, and almost insoluble in acetone. The substance melts at $201-2^{\circ}$ to a liquid filled with bubbles.

Kjeldahl: 0.1163 g. subst.; 18.15 cc. 0.1 N HCl.

Calc. for $C_9H_{11}O_2N_3$: N, 21.76%. Found: N, 21.86%.

m-Chloroacetyluraminophenylacetamide, *m*- $ClCH_2CONHCONHC_6H_4CH_2CONH_2$.—The urea was chloroacetylated in molten chloroacetic acid by means of chloroacetyl chloride as in previous examples. After heating for one-half hour on the water bath the melt was poured into water, precipitating the chloroacetyl derivative as a gum which soon crystallized. This was filtered off, ground up in a mortar with sodium acetate solution until neutral to Congo red, and recrystallized twice from 50% alcohol, forming slightly pinkish aggregates or minute plates and flat needles. When rapidly heated to 175° , then slowly, it melts at $179-81^{\circ}$ with gas evolution and preliminary softening. The amide is soluble in the cold in acetic acid, sparingly in acetone or 95% alcohol, and is soluble in hot water or 50% alcohol. It gives the Beilstein test.

Kjeldahl: 0.1514 g. subst.; 16.75 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{12}O_3N_3Cl$: N, 15.59%. Found: N, 15.50%.

p-Uraminophenylacetamide.—20 g. *p*-aminophenylacetamide¹ were suspended in 400 cc. water and 67 cc. double normal hydrochloric acid added. To the resulting solution was slowly added a solution of 11.5 g. potassium cyanate, keeping the temperature below 5°. The urea separated in a yield of 22 g. Recrystallized from water, it forms transparent, rectangular plates which dissolve in boiling water and are scarcely soluble in boiling alcohol or acetone. When rapidly heated to 230°, then slowly, it softens, melts with effervescence at 233–6° and then resolidifies.

Kjeldahl: 0.1297 g. subst.; 20.0 cc. 0.1 *N* HCl.

Calc. for $C_9H_{11}O_2N_3$: N, 21.76%. Found: N, 21.60%.

p-Chloroacetyluraminophenylacetamide.—The urea was chloroacetylated as in previous examples. After one-half hour the melt was poured into water, precipitating the chloroacetyl compound in crystalline form. Recrystallized from 95% alcohol it forms aggregates of delicate needles which are very difficultly soluble in the cold in the usual solvents and less sparingly soluble in hot alcohol or acetic acid than in the others. When rapidly heated to 195°, then slowly, it melts and decomposes at 200–1°, with preliminary softening.

Kjeldahl: 0.1539 g. subst.; 17.0 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{12}O_3N_3Cl$: N, 15.59%. Found: N, 15.47%.

p-Uraminophenoxyacetamide.—30 g. *p*-aminophenoxyacetamide (p. 251) were dissolved in 400 cc. water and 50 cc. acetic acid and treated, with cooling, with a solution of 14.6 g. potassium cyanate. The urea separated in quantitative yield on shaking. Recrystallized from water containing a little ammonia it forms radiating masses of long, flat, slightly brownish needles which are less difficultly soluble in boiling water than in the other usual neutral solvents. When rapidly heated it softens above 210°, melts with gas evolution at about 230°, resolidifies partly, and melts again completely at 260°.

0.1111 g. subst.; 19.8 cc. N, 759 mm., 27.5°.

Calc. for $C_9H_{11}O_3N_3$: N, 20.09%. Found: N, 20.22%.

¹ *Loc. cit.*

p-Chloroacetyluraminophenoxyacetamide. $\text{ClCH}_2\text{CONHCONHC}_6\text{H}_4\text{OCH}_2\text{CONH}_2$.—20 g. of the urea were dissolved in 60 g. molten chloroacetic acid and treated with 10 cc. chloroacetyl chloride. After warming for 15 minutes on the water bath the melt was poured into water. The yield of chloroacetyl derivative was 14 g. Recrystallized from acetic acid it forms masses of minute needles which are practically insoluble in boiling 95% alcohol or acetone but dissolve readily in hot acetic acid. When rapidly heated to 225° , then slowly, the substance softens and darkens, decomposing at 230° .

0.1372 g. subst.; 17.9 cc. N, 759 mm., 28.0° .

Calc. for $\text{C}_{11}\text{H}_{12}\text{O}_4\text{N}_3\text{Cl}$: N, 14.72%. Found: N, 14.78%.

The following two substances were prepared in an attempt to obtain *p*-chloroacetyluraminophenoxyacetamide by an indirect method. The final step was not carried out, however, owing to the satisfactory outcome of the direct chloroacetylation of *p*-uraminophenoxyacetamide.

p-Uraminophenoxyacetic Methyl Ester, $p\text{-H}_2\text{NCONHC}_6\text{H}_4\text{OCH}_2\text{CO}_2\text{CH}_3$.—9 g. *p*-aminophenoxyacetic methyl ester hydrochloride¹ were dissolved in 100 cc. water and treated, with cooling, with a solution of 3.4 g. potassium cyanate. The urea separated in a yield of 6.3 g. Recrystallized, with bone-blackening, from 50% alcohol it forms almost colorless crystals which dissolve more readily in boiling 50% alcohol than in boiling water or 95% alcohol and are also difficultly soluble in methyl alcohol or acetone. When rapidly heated to 190° , then slowly, it melts at $192\text{--}3^\circ$ with slow gas evolution.

Kjeldahl: 0.1092 g. subst.; 9.70 cc. 0.1 N HCl.

Calc. for $\text{C}_{10}\text{H}_{12}\text{O}_4\text{N}_2$: N, 12.50%. Found: N, 12.45%.

p-Chloroacetyluraminophenoxyacetic Methyl Ester.—9 g. of the uramino ester were boiled under a reflux condenser for about 6 hours in 90 cc. of dry benzene with 4 cc. chloroacetyl chloride. The mixture was cooled, treated with water, filtered, washed with benzene and water, and dried. The yield of chloroacetyl derivative was 11 g. Recrystallized from amyl alcohol it forms flat, slightly brownish prisms which soften above 170° and melt at $181\text{--}3^\circ$ with gas evolution. The

¹ *Loc. cit.*

substance dissolves readily in hot acetic acid, less easily in hot amyl alcohol and only sparingly in boiling acetone or chloroform.

Kjeldahl: 0.1993 g. subst.; 13.30 cc. 0.1 *N* HCl.

Calc. for $C_{12}H_{13}O_5N_2Cl$: N, 9.32%. Found: N, 9.35%.

(D) *Ureas with Other Substituents and Side Chains.*

The following substances were prepared in an attempt to synthesize *p*-hydroxyphenylchloroacetylurea, *p*- $HOC_6H_4NHCONHCOCH_2Cl$. In an experiment on the direct chloroacetylation of *p*-hydroxyphenylurea both the urea and hydroxyl groups were chloroacetylated and attempts were then made to cover the hydroxyl group by acetylation. Treatment of *p*-hydroxyphenylurea with acetic anhydride and sulfuric acid resulted in the acetylation of both the urea and hydroxyl groups, but acetylation of the hydroxyl group alone was finally accomplished by the pyridine method. The resulting *p*-acetoxyphenylurea could be smoothly chloroacetylated and the product so obtained was as satisfactory for the synthesis in which it was required as the free hydroxy compound originally sought.

p-Chloroacetyluraminophenyl Chloroacetate, *p*- $ClCH_2CONHCONH-C_6H_4OCOCH_2Cl$.—5 g. *p*-hydroxyphenylurea were heated under a reflux condenser with 25 cc. benzene and 8 g. chloroacetylchloride as in previous examples. The mixture was finally boiled up with about 200 cc. benzene, filtered from tar, and the product obtained on cooling (2.2 g.), recrystallized twice from alcohol, forming delicate needles and long, thin plates which melt at $185.5-7.5^\circ$ with preliminary softening and resolidify on cooling. The substance dissolves in the cold in acetone, on boiling in water, alcohol, or chloroform, and is sparingly soluble in benzene. It gives a strong test for halogen.

Kjeldahl: 0.1541 g. subst.; 10.2 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{10}O_4N_2Cl_2$: N, 9.18%. Found: N, 9.27%.

Diacetyl-p-hydroxyphenylurea, *p*- $CH_3COOC_6H_4NHCONHCOCH_3$.—4.5 g. *p*-hydroxyphenylurea were treated with 10 cc. acetic anhydride and a few drops of concentrated sulfuric acid. After finally heating on the water bath for a few minutes the solution was cooled and shaken with water. The resulting solid was recrystallized from 50% alcohol, yielding 6 g. of the diacetyl derivative. Recrystallized

again from ethyl acetate it forms delicate, pinkish needles which melt at $213.5-14^{\circ}$ (cor.). It is more readily soluble in the cold in chloroform than in the other usual neutral organic solvents, but also dissolves in the others on warming.

Kjeldahl: 0.2089 g. subst.; 17.7 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{12}O_4N_2$: N, 11.87%. Found: N, 11.87%.

p-Acetoxyphenylurea.—45 g. *p*-hydroxyphenylurea were dissolved in 350 cc. pyridine, cooled to -5° , and treated, with shaking and cooling, with 25 cc. acetyl chloride, added drop by drop. The mixture was then allowed to warm up to room temperature and was poured into a mixture of ice and 25% sulfuric acid. The resulting precipitate was filtered off, washed thoroughly with water, and dried. The yield was 45.2 g. Recrystallized from 95% alcohol the acetate forms felted needles which melt to a brown liquid at $201-2.5^{\circ}$ with slight gas evolution and preliminary softening. It is soluble in boiling water or alcohol and almost insoluble in boiling benzene.

Kjeldahl: 0.1607 g. subst.; 16.50 cc. 0.1 *N* HCl.

Calc. for $C_9H_{10}O_3N_2$: N, 14.43%. Found: N, 14.38%.

p-Acetoxyphenylchloroacetylurea, *p*- $CH_3COOC_6H_4NHCONHCOCH_2Cl$.—21 g. of the acetoxyurea were boiled under a reflux condenser for several hours with 210 cc. benzene and 13.4 g. chloroacetyl chloride. In order to complete the crystallization of the chloroacetyl derivative ligroin was added on cooling. After recrystallization from 50% alcohol the yield was 19.5 g. Recrystallized again from 95% alcohol it forms almost colorless spindles and plates which melt at $181-2.5^{\circ}$ (cor.) with preliminary softening. The substance is easily soluble in hot 95% alcohol, sparingly in the cold, and is quite soluble at room temperature in acetic acid.

Kjeldahl: 0.2007 g. subst.; 15.0 cc. 0.1 *N* HCl.

Calc. for $C_{11}H_{11}O_4N_2Cl$: N, 10.36%. Found: N, 10.47%.

p(?)-Uraminohloroacetylbenzylamine, *p*- $H_2NCONHC_6H_4CH_2NHCOCH_2Cl$.—13.6 g. phenylurea were dissolved in 100 g. sulfuric acid and treated with 12.3 g. methylolchloroacetamide, according to Einhorn's method for introducing the $CH_2NHCOCH_2Cl$ group into aromatic

compounds.¹ After 2 days the solution was poured onto ice, yielding a gummy precipitate which gradually crystallized. After recrystallizing from alcohol the yield was 10 g. The substance forms microscopic spears, which are almost insoluble in hot toluene or acetone, sparingly soluble in hot ethyl acetate, and readily so in boiling alcohol. When slowly heated above 170° it melts with preliminary softening at 173–4° to a yellow liquid, a higher figure being obtained if the heating is more rapid.

Kjeldahl: 0.1487 g. subst.; 18.40 cc. 0.1 *N* HCl.

Hydrolysis: 0.2548 g. subst.; 0.1475 g. AgCl.

Calc. for $C_{10}H_{12}O_2N_3Cl$: N, 17.40%; Cl, 14.67%. Found: N, 17.33%; Cl, 14.33%.

p(?)-Uramino- ω -chloroacetophenone, *p*- $H_2NCONHC_6H_4COCH_2Cl$.—35 g. of anhydrous aluminium chloride were added, with shaking, to a suspension of 10 g. phenylurea in 75 g. of dry carbon bisulfide. To the mixture were added, drop by drop, 9.5 g. chloroacetyl chloride, finally warming on the water bath until the reaction was over. Ice was added and the whole vigorously shaken until decomposition was complete. Recrystallized from 95% alcohol the ketone forms cream-colored, nacreous plates which melt constantly at 197–8° with decomposition. The yield was 7 g. The compound is soluble in acetone or hot alcohol and only sparingly in hot benzene.

Kjeldahl: 0.1674 g. subst.; 15.75 cc. 0.1 *N* HCl.

Hydrolysis: 0.1661 g. subst. 0.1105 g. AgCl.

Calc. for $C_9H_9O_2N_2Cl$: N, 13.18%; Cl, 16.68%. Found: N, 13.18%; Cl, 16.45%.

p-Aminophenoxyethyl Bromide (*p*-Aminophenyl Bromoethyl Ether) Hydrobromide.—*p*-Acetaminophenoxyethyl bromide was smoothly hydrolyzed by boiling for one hour under a reflux condenser with 5 parts of hydrobromic acid (d. 1.49). The *p*-aminophenoxyethyl bromide hydrobromide separated on cooling and was filtered off and washed with a little acetone. A portion was recrystallized, with bone-blackening, from a little water containing a few drops of hydrobromic acid, forming thin, glistening plates which melt at 227–8° with gas evolution and preliminary softening. It is difficultly soluble in absolute alcohol at room temperature, but dissolves in water. An

¹ *Loc. cit.*

aqueous solution gives a slowly-developing, purple color with ferric chloride. The salt is readily diazotized.

0.2106 g. subst.; 8.70 cc. N, 757 mm., 26.5°.

Calc. for $C_8H_{10}ONBr.HBr$: N, 4.72%. Found: N, 4.69%.

p-Aminophenoxyethyl Bromide, $p-H_2NC_6H_4OCH_2CH_2Br$.—A portion of the crude hydrobromide was dissolved in water and made alkaline with sodium carbonate solution. The bromide separated as an oil which crystallized almost immediately. The product was quickly filtered off, washed with water, and recrystallized, with bone-blackening, from a little 95% alcohol, forming minute, glistening platelets which are readily soluble in acetone or chloroform, less easily in benzene or alcohol. The bromide melts with preliminary softening at 83–4.5° to a liquid which immediately becomes turbid and decomposes without clearing at about 260°.

Kjeldahl: 0.1958 g. subst.; 9.25 cc. 0.1 N HCl.

Calc. for $C_8H_{10}ONBr$: N, 6.48%. Found: N, 6.62%.

p-Uraminophenoxyethyl Bromide (*p*-Uraminophenyl Bromoethyl Ether), $p-H_2NCONHC_6H_4OCH_2CH_2Br$.—This substance was obtained by reacting crude *p*-aminophenoxyethyl bromide hydrobromide with potassium cyanate in aqueous solution. Recrystallized, with bone-blackening, from 95% alcohol it forms aggregates of flat needles which melt slowly at 160–2° with preliminary softening. It is quite soluble in alcohol or acetone at room temperature, sparingly in ether or chloroform.

Kjeldahl: 0.1501 g. subst.; 11.70 cc. 0.1 N HCl.

Hydrolysis: 0.1152 g. subst.; 0.0828 g. AgBr.

Calc. for $C_9H_{11}O_2N_2Br$: N, 10.82%; Br, 30.84%. Found: N, 10.92%; Br, 30.58%.

STERILIZATION AND CLOSURE OF SUPPURATING FRACTURES.

BY M. GUILLOT, M.D., AND H. WOIMANT, M.D.

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During the past year, it has been shown in this hospital that recent fractures can be sterilized and closed (1). It was important to ascertain whether the same method could be applied to older cases in hospitals removed from the battlefield, and for this purpose old and highly infected fractures were removed from the trains bound for Paris at a station situated close to our hospital. The patients reached the hospital a few hours earlier than they would have reached the Paris hospitals. But this anticipation of a few hours was negligible, since 2 to 46 days and even 8 months had elapsed since the injury.

A great many fractures of the long bones remain fistulous. Probably 50 per cent of fractures of the thigh still suppurate after ten months' treatment. This estimate of the number of osseous lesions which remain fistulous after long deferred treatment may appear excessive to surgeons primarily concerned with industrial traumatism. But unfortunately in the actual conditions of war surgery, i. e., fractures of the long bones produced by bursting shells, our estimate falls short of the reality.

After four months of experimentation devoted to regulating the details of this special application of Carrel's method, we are in a position to affirm that fractures in full process of suppuration can be sterilized and closed. The technique comprises the following points: (a) preliminary disinfection, (b) operative treatment, (c) sterilization, and (d) closing of the focus of fracture.

Preliminary Disinfection.

In nearly all instances the patients had received surgical treatment a few hours after injury in a hospital near the front. At first sight this treatment appears either complete or incomplete. In some cases

we are confronted with a widely exposed fracture; in others with a badly cleansed cavity which communicates with the exterior only by means of one or more inadequate openings. In the latter case one is tempted to perform an immediate operation, and this procedure has often been followed in the base hospitals. But this should be absolutely prohibited, for an operation conducted under these conditions is followed by a marked rise in temperature, and sometimes by septicæmia and death.

Before an operation is attempted, infection must be reduced as much as possible. This is accomplished by discontinuous flushing with Dakin's solution. If it is impossible to introduce tubes into the focus, careful incisions may be resorted to, but the bones should not be handled. These incisions do not expose the patient to the grave complications which ordinarily follow osseous operations performed in an infected focus. This preliminary disinfection must be done in all cases, whether the previous operation has been well or badly conducted. An exception may be made in the case of very old fistulous fractures which show no inflammatory reaction.

The first process must be carried out by an exact observance of the principles of Carrel's method (2) and of the details of its application. The progress of disinfection should be followed on the bacteriological curve, for it is imperative that one should be able to visualize at a glance the modifications in the number of bacteria. Every other day, after the flushing has been suspended for two hours, films are made of secretions from the wound taken from such parts as are apparently most highly infected. The bacteria of three microscopic fields are counted on this preparation and the average result of the observations is registered on the curve. The curves thus obtained are different from the sterilization curves of fresh fractures. The latter, at the outset, show no or very few bacteria in films of their secretions, and the bacteria appear in large quantities only after twenty-four or thirty-six hours. They increase in number for some time, after which a rapid and regular diminution occurs. This is illustrated by the following experiment.

Case 1.—Recent, comminuted bullet fracture of the femur. Immediate removal of the splinter of bone; rapid sterilization. Suture with horsehair on the fourteenth day.

G. M., age 42; wounded July 1, 1916, at 11:30 p.m.; entered Hospital 21 six hours later.

Examination.—Small opening in the center of the antero-external region of the left thigh. No outlet. Abnormal mobility due to a fracture of the center of the femoral diaphysis. Moderate inflammation. The roentgenogram shows a highly splintered fracture, with a bullet in the center (Fig. 1).

Operation.—Ether anaesthesia; resection of the edges of the cutaneous and aponeurotic openings. Wide incision; ablation of a small number of free splinters; preservation of several adherent splinters.

Flushing every two hours with Dakin's solution by means of four tubes. Continuous extension.

As is usual in recent wounds, the first examination failed to reveal any bacteria (Fig. 3). Two days later one bacterium was found in every ten microscopic fields. The maximum (twenty bacteria per field) was reached on the ninth day. On the thirteenth day the wound was sterile. On the fourteenth day the wound was sutured with horsehair and without drainage.

The two roentgenograms taken at the beginning and end of the treatment (Figs. 1 and 2, respectively) show to what extent it was possible to reduce the fracture after its reunion.

The bacteriological curves observed in the case of old fractures are usually very different from the one we have just examined. In these cases, the beginning of the curve indicates the presence of an infinite number of bacteria, except where a dry scab makes it impossible to obtain a film of the secretions. On the other hand, the diminution in the number of bacteria takes place more slowly than in the case of recent fractures, and is accompanied by oscillations which may last for some time, as in the following case.

Case 2.—Old infected shell fractures of the sacrum. Slow preliminary disinfection. Hollowing out of the site of fracture. Sterilization. Closing of wound over an adipose graft.

P. B., age 34; wounded September 17, 1916. Operation by incision and extraction of splinters in a front-line ambulance thirty hours later. Entered Hospital 21 October 5, eighteen days after being wounded.

Examination.—In the sacral region was a large horizontal, anfractuous wound, 12 by 6 centimeters, communicating (a) by means of a submuscular sinus with a small wound in the left buttock, and (b) with the sacral fracture. The entire visible portion of the wound was covered with a dry scab produced by the local application of phenol. Lymphangitis. Temperature 37.6°C. General condition satisfactory. Films showed twenty to forty bacteria per field.

Preliminary disinfection by flushing every two hours with Dakin's solution was immediately started. The curve (Fig. 4) shows that the films made from the

dry scab of the wound contained only a small number of bacteria. Not until the third day did the bacteria become innumerable. After a period of oscillation, the curve appeared from October 21 to 29 to tend toward sterilization. But this was followed by a series of fluctuations of five to twenty bacteria, showing the presence in the wound of foreign septic bodies (sequestra, fragments of clothing, etc.). Under these conditions intervention became necessary.

November 12, incision of the sinus. Opening of the site of osteitis, which was seen to contain six small sequestra and to communicate with the sacral canal. All the suspected osseous tissue was resected with the gouge-forceps (*pince-gouge*). Beneath the left sacrolumbar muscles muscular detachment was found. This was opened and the wall excised. All the visible necrotic tissue was carefully removed from the wound (Fig. 5).

Sterilization by means of flushing was next resumed. The day after the operation, the temperature rose to 39°C. and the number of bacteria increased. The temperature fell rapidly, and on November 18, six days after the operation, the wound was sterile.

For several days a pulmonary complication made it impossible to close the wound. Finally, on December 15, after a short reinfection followed by a return to the sterile condition, the wound was filled up by means of an autoplasty (Fig. 6), after an adipose graft in the focus of the fracture of the sacrum had been effected. The graft was taken from the left buttock.

On the sixth day, as a result of traction of the flaps, three of the stitches were cut. The adipose graft in the wound was living and was rapidly becoming covered with fine granulations and later with epidermis.

This observation of a highly infected fracture of the sacrum, the sterilization of which proceeded exceptionally slowly, gives an exact idea of the method followed. It will be seen that the preliminary disinfection was patiently pursued for as long a time as the bacteriological curve continued to decline. As soon as a plateau appeared in the chart curve, surgical intervention was seen to be necessary. When this had been performed, the course of sterilization continued in a normal manner.

We have said that it was not always necessary to have recourse to surgical intervention in order to secure the sterilization of an old fracture. This is explained by the fact that the operation performed at a first-line ambulance may have been sufficient. Case 3 is an example of this type.

Case 3.—Infected fracture of the thigh, nine days old. Sterilization attained on the twelfth day. Closure on the seventeenth day, without operation.

L. J., age 25; wounded October 11, 1916; operated upon the following day in an ambulance on the Somme. October 20, entered Hospital 21.

Examination.—Fracture of the middle of the left thigh bone. Two wide wounds led to the site of fracture. Their long axis was longitudinal. The outer wound measured 12 by 6 centimeters; the inner had similar dimensions (Fig. 7). Abundant suppuration; pyocyanic. Extensive inflammation of the limb. Patient looked tired. Films showed forty to sixty bacteria per field.

Figure 9 shows a splintered fracture with fissures which descended very far down and extended almost to the top of the limb. Extensive loss of substance over an area of 8 centimeters. However, as the fragments came in contact with one another, the continuity of the osseous layer was uninterrupted.

Flushing every two hours with Dakin's solution. Exploration conducted at the time that the tubes were inserted showed that the upper end of the fracture was exposed and that a medullary plug was already covering the lower extremity. The roentgenogram showed that only the upper part of the fracture was being flushed. As a matter of fact, the disposal of the tubes was good, for sterilization was obtained in twelve days (Fig. 10) and on the seventeenth day the fracture was closed.

Closure on November 6. Both wounds were closed; the inner one was sewed with horsehair in one single cutaneous plane; the outer in two planes over a filling of Beck's paste. Of these two planes, the first, or musculo-aponeurotic plane, was sewed with catgut; the second, or cutaneous plane, with horse-hair (Fig. 8).

Operative Treatment in the Focus of the Fracture.

It must be assumed at the outset that the appearance of a plateau in the bacteriological disinfection curve is an indication of the presence in the wound of infected foreign bodies. These may be necrotic fragments of tendon, aponeurosis or bone, projectiles, particles of clothing, etc. Of whatever nature, it is imperative that the foreign body should be removed.

When surgical treatment is applied to flat bones or epiphyses of long bones, it presents no particular difficulty. With the help of a gouge-forceps or a *fraise* all the compact or spongy tissue affected with osteitis or preventing extensive flushing is carefully abraded. It is necessary, as far as possible, to avoid opening the joints which are capable of movement, without losing sight of the fact that any part which is well exposed and thoroughly flushed will never become dangerously infected.

The surgical treatment of old fractures of the long bones, on the other hand, presents real technical difficulties. It does not suffice to remove the sequestra, or to abrade the necrotic surfaces, or even to

curette all the loci of osteitis. This form of surgery, which has been practiced on thousands of cases since the beginning of the war, has usually resulted in failures. As a rule, when this method has been used, infection sets in rapidly in the bone marrow and in the clots of blood remaining in the wound. Osteomyelitis and lamellar necrosis of the walls of the medullary canal are the ordinary consequences of this kind of surgery, while opening of intra-osseous abscesses produces fresh fistulæ.

In order to avoid these complications, it is necessary to perform a systematic operation involving the successive consideration of the following factors: incision, periosteal callus, sequestra, splinters, and bone ends.

Incision.—In preparing the patient, tincture of iodine should not be employed. Dakin's solution, which is necessary for the further sterilization of the wound, generally produces burns on an iodized surface.

The incision need not necessarily extend beyond the fistula or the wound of the first operation. Exposure of each diaphysis is made according to definite rules, by which a maximum portion of the bone may be exposed with a minimum of risk to the adjacent organs. The operation should be conducted plane by plane, by successively detaching and utilizing each in such a manner that the hæmostasis may be facilitated and the wound as a whole may contract from the surface inward. If the line followed is that of a fistula or of an infected wound, it is preferable to excise all the infected and sclerous tissues, and the process of elimination or sterilization will thereby be reduced. But it is important to keep account of the quantity of tissue disposed of, and care must be taken that sufficient skin is kept for future closing. The consideration of this fact has often prevented us from excising the edges of the mouth of a fistula.

Osteitis of the Periosteal Callus.—The best method of penetrating the periosteal callus, after the surrounding osteogenic membrane has been turned back, is effected by careful manipulation of the rugine. With the help of roentgenograms, this uncovering must be confined to that part of the callus which is to be excised. Through the opening thus made, a gouge-forceps is introduced, which extends the infected cavity of the periosteal callus in the direction of the bone ends

which are to be explored. The extremities of the fragments must be exposed. In order to reach the end of the callus, it will suffice to scoop out with a curette every part affected with osteitis.

If the fracture is recent, or if the periosteum has suffered extensive destruction, there is no callus. In such cases the fracture cavity can be immediately penetrated, and the bone ends are consequently more easily accessible.

Sequestra.—These may be found in the muscles, focus of fracture, and medullary canal. If present in the muscles they are easily removed by an incision; but they are harder to find if situated on the side opposite from which the incision is made. In such cases, after making a good roentgenogram, they are best reached by means of a special incision. A careful investigation should be made for sequestra which may have been projected into the medullary canals at the time of injury. Their presence constitutes one of the causes for the absence of the medullary plug observed in the course of the consolidation of fractures.

Splinters.—If the splinters are necrotic, all fragments which are not indispensable to consolidation must be removed.

Bone Ends.—These are sometimes exposed at the site of fracture, and sometimes obliterated by an osseous plug. The sealing of the fragments by the medulla must be considered as a normal stage in the evolution of open fractures. This process is impeded in various ways. Sometimes, as we have seen, the projection of a fragment of bone, which rapidly becomes necrosed, may be the cause; in other cases the plug, after formation, has been destroyed by osteitis. But in the majority of cases the defect of obturation is explained by an intense infection, which has from the outset produced necrosis of the contents of the medullary canal and of its walls. In such cases the bone ends are laid bare and are seen to be exposed and bathed in the pus proceeding from the site of fracture.

All severe accidents that follow upon operation in the focus of infected fractures are invariably connected with the opening of the medullary canal. The operation sets free the bacteria and provides them with contused tissues and clots of blood favorable to their cultivation, with the result that if in their vicinity there is an inadequately drained canal filled with an easily infectible marrow, the immediate result will be the occurrence of severe septic accidents.

We have regularly observed the following phenomena: (a) A fracture in which the bone ends are obturated causes no postoperative reaction. (b) A fracture in which the bone ends are not occluded produces an intense reaction, if the cavity of the medullary canal is not plentifully flushed by the antiseptic solution. (c) A fracture in



FIG. 1 (at left). Roentgenogram, Case 1, showing fracture of left femur, with bullet in the center.

FIG. 2. Roentgenogram taken at the end of treatment in Case 1.

which the non-occluded bone ends have been widely opened by surgical intervention will produce no reaction.

From the above facts we have drawn the following conclusions for the treatment of bone ends: (1) The surface of the medullary plugs should be explored with a curette, in order to ascertain that there is no communication with the subjacent medullary canal and that no

sequestrum is enclosed. (2) The medullary canals which have remained open should be hollowed out with a *pince-gouge* in such a form as to produce a wedge-shaped cavity, communicating freely with the flushing tubes.

As an illustration, we shall report two cases of fracture of the humerus, one of which was characterized by two obturated medullary canals, and the other by the fact that the bone ends remained opened.

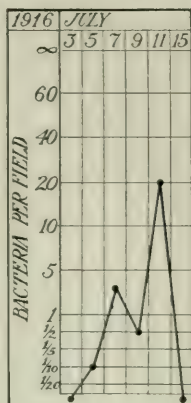


FIG. 3. Bacteriological curve of films from the wound in Case 1.

Case 4.—Infected shell fracture of the upper third of the humerus. Double medullary plug. Operative treatment without resulting reaction. Closure of wound.

V. M., age 21 years. Wounded October 21, 1916. Operated on the following day in a first-line ambulance; entered Hospital 21 November 6, seventeen days after sustaining the injury.

Examination.—Comminuted fracture of the surgical neck of the left humerus (Fig. 11). Three wounds: anterior, posterior, and external (Fig. 12).

Two drains crossed the site of fracture from front to back. Abundant supuration; slight œdema of the member. General condition good. Films showed an innumerable number of bacteria in the anterior wound, and about thirty per field in the posterior wound.

Discontinuous flushing with Dakin's solution. The number of bacteria diminished in both wounds, but the curve soon indicated a plateau, at the degree of infection of twenty bacteria per field (Fig. 14).

Operation.—December 2. Anterior incision made in the wound. Removal of a sequestrum situated above, two sequestra situated laterally, and the necrotic end of the lower fragment. The two bone ends were obturated by a medullary plug (Fig. 15).

Temperature 37.9°C. on the evening of the second day. Rapid decline in the number of bacteria, and sterilization effected twelve days later. Suture with horsehair in December 21 (Fig. 13).

Case 5.—Infected shell fracture of the upper third of the humerus. Absence of medullary plug at the site of the upper fragment. Operation followed by an inflammatory reaction. Closure of wound.

C. D., age 28; wounded October 11; operated on the same day in a first-line ambulance. Entered Hospital 21 October 20, nine days after receiving injury.

Examination.—Punctured wound in the anterior region of the left axilla. At the level of the upper third of the arm there was a vertical posterior wound, measuring 8 by 6 centimeters (Fig. 16). The surface of this wound was covered with necrotic tissue. The wound led into a focus of splintered bones occupying the lower part of the surgical neck of the humerus (Fig. 18). Œdema. General

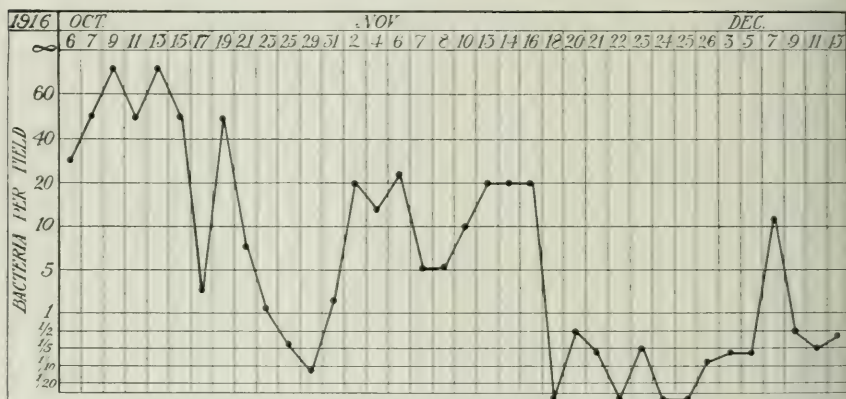


FIG. 4. Bacteriological curve of films from the scab of the wound in Case 2.

condition good. Temperature 38°C. Films taken of the posterior wound showed ten bacteria per field. Flushing every two hours with Dakin's solution.

On November 5, after some fluctuation (Fig. 19), the number of bacteria had diminished to less than one in twenty fields. But as cicatrization had been very rapid, the wound had become narrow and it was impossible to flush it out. The bacteria immediately increased in number until they reached forty to sixty per field. It was evident that under these conditions, the fracture could not be sterilized without surgical operation.

Operation.—On November 11, excision *en masse* of the fistula. This led to a small opening at the back of the periosteal callus. The callus was opened with chisel and hammer. The gouge-forceps was enlarged in such a manner as to expose the bone ends. The callus contained three small longitudinal sequestra. The medullary canal of the upper end of the bone remained open. Its edge was abraded sufficiently to permit flushing. The extremity of the lower fragment

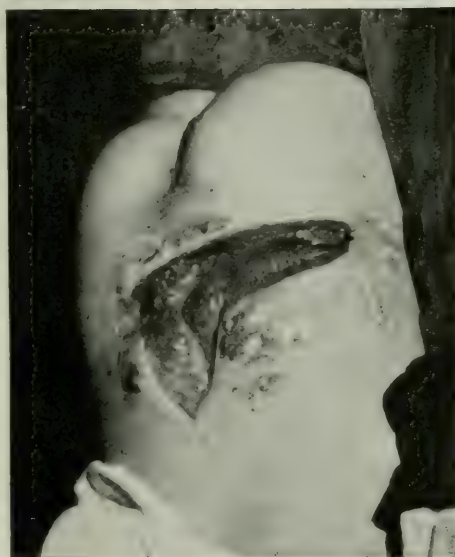


FIG. 5. Sacrolumbar wound in Case 2.

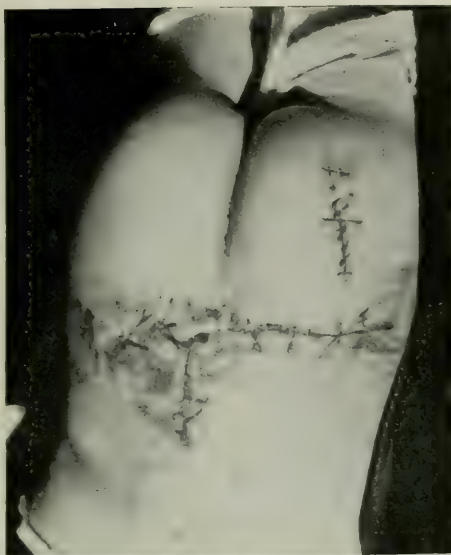


FIG. 6. The wound was filled with an autoplasty after an adipose graft in the sacrum.

was obturated by a medullary plug, but the edge of the bone was necrosed for a distance of several millimeters. This bone was removed with the gouge-forceps.

The operation was succeeded by a fairly strong inflammatory reaction, and the following day the temperature rose to 38.9°C. But the normal condition was speedily restored, and when the temperature fell, films taken on November 15 showed that the number of bacteria had already been reduced to eight per field.

Closure.—On December 3, the focus was sterilized. On the 7th it was closed over a chloramine and sodium stearate paste filling, by means of two planes of suture, one musculo-aponeurotic, the other cutaneous (Fig. 17).

Sterilization of the Focus of Fracture.

In infected fractures of the long bones, the wide opening of the non-obturated medullary canal is a factor of importance. But this detail in itself does not suffice to prevent every form of reaction. It is usually observed that the temperature reaches its maximum in the evening of the second day after the operation. Thus, there is an interval of more than twenty-four hours during which the multiplication of bacteria at the surface of the new wound should be prevented as much as possible.

It was found that in the first days of sterilization the most infected parts were always the blood clots situated in the vicinity of the severed bones or of the medullary cavities. Therefore, it was attempted to prevent the formation of the clots. With this object in view, after having clamped and ligated all the bleeding vessels with the utmost care, we arrested all oozing of blood from the bones and medulla, by means of prolonged flushing with physiological salt solution at 40°C. Sometimes more than a quarter of an hour was needed to obtain this result, but there is no doubt but that this perfecting of the technique has shortened the time employed in the process of sterilization.

In a similar manner the process of disinfection during the first two days was accelerated by flushing out the wound every half hour during the day and every hour during the night, after which the usual flushing every two hours was resumed.

When flushing is repeated at close intervals, it is important to use hypochlorite prepared electrolytically, as this substance exerts only slight irritation on the skin.



FIG. 7. Wound of the thigh, Case 3.



FIG. 8. The same as Fig. 7, closed with horsehair.

The combination of these three processes, the wide opening of the medullary canals, careful hæmostasis, and frequent flushing with electrolytic hypochlorite, has enabled us practically to suppress all post-operative febrile reactions.

This method of procedure has also greatly affected the course of the bacteriological curve. After our first operations on old fractures, the

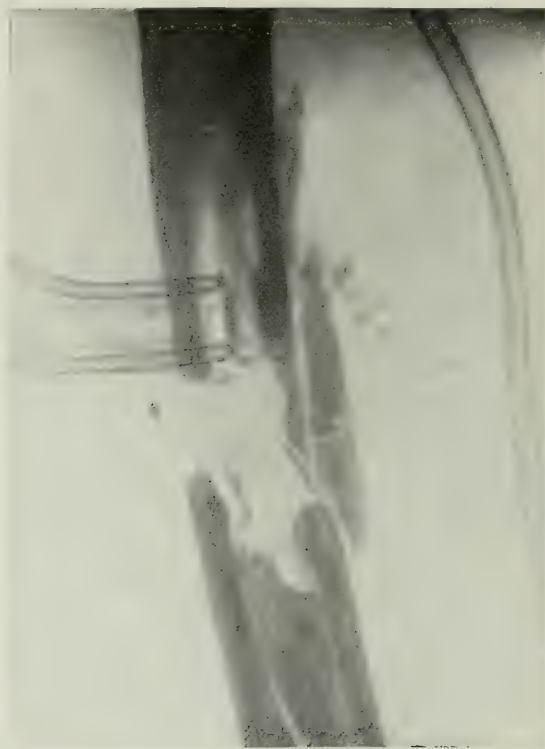


FIG. 9. Roentgenogram Case 3, showing fracture of left femur with deep fissures.

number of bacteria increased *ad infinitum* during the next few days. It will be seen by means of the accompanying charts that this bacterial increase has now become almost negligible. At all events, after a varying length of time, the number of bacteria falls below three per field. When this stage is reached, it is advisable to let several days elapse before closing the wound.

Closure of the Wound.

In fresh fractures, once sterilization is assured, the closing of the cavity is effected without any special difficulty. After resection of the edge of the epidermis, the edges of the wound are dissected. Profuse bleeding is avoided as much as possible. Horsehair sutures are performed. If pressure is applied to the dressing, while at the same time the bone ends and adherent splinters are brought together, the cavity produced by the fracture will be completely obliterated.

The same procedure is also successful in old fractures if the periosteal callus is not in process of formation, or if the condition of the muscles make it easy to force them back into the cavity.

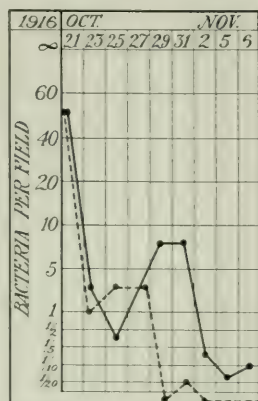


FIG. 10. Bacteriological curve of films from wounds, Case 3.
 ———, external wound; -----, internal.

In all other cases the focus of fracture cannot be closed without the aid of a filling, for otherwise a cavity would be left beneath the sutures, in which the secretions of the wounds would accumulate. Various kinds of filling were experimented with: Mosetig's substance, Beck's paste (Case 3), chloramine paste (Case 5), and adipose grafts (Case 2).

The results can be summed up in the following statements: Beck's paste is superior to that of Mosetig, for it is easier to apply and involves no danger of intoxication. Beck's paste, used under conditions with which we deal, necessitates the interposition of a musculo-

aponeurotic plane between the paste and the cutaneous sutures. In the case of old fractures it is hard to effect this interposition. The chloramine paste has the advantage that it can be applied directly beneath cutaneous sutures. But it falls away if, as frequently occurs in the case of old fractures, the sutures pull and gap slightly, owing to

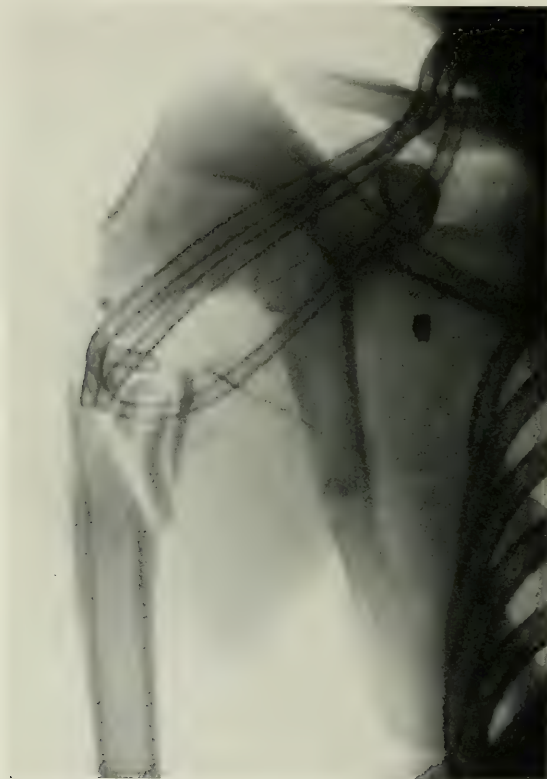


FIG. 11. Roentgenogram Case 4 showing comminuted fracture of the surgical neck of the left humerus.

the lack of tissue. The best filling seems to be an adipose graft; but this procedure must be limited to cases in which the formation of the periosteal callus is well advanced and where there is no danger of pseudarthrosis. With this one limitation, this method is found suitable in the majority of cases, since (1) it does not require the making of

a musculoaponeurotic layer; (2) the graft is always obtainable in the vicinity of the zone of operation; and (3) it will keep in place even in cases where, owing to the lack of a sufficient quantity of cutaneous tissue, the wound has remained partially open.

Case 6.—Old infected shell fracture of the tibia. Sterilization of the focus. Wound closed over an adipose graft.

J. V., age 31; wounded October 10, 1916; operated on fifty-one hours later in an ambulance on the Somme; entered Hospital 21 October 20, ten days after being injured.

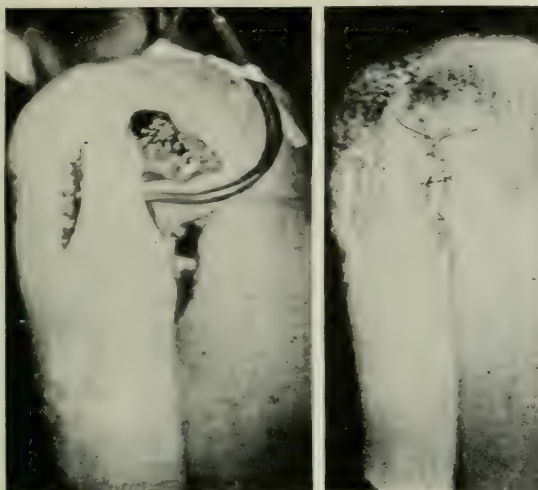


FIG. 12 (at left). Anterior, posterior, and external wounds, Case 4.

FIG. 13. Wounds sutured with horsehair.

Examination.—The inner surface of the leg and of the right thigh showed a large muscular wound, of about 50 square centimeters in area (Fig. 20). At the lower third of the wound there was extensive loss of tibial tissue (Fig. 22). The muscles were covered with necrotic tissue. The focus of fracture contained a serous fluid, as well as the broken ends of the bone, which constituted an irregular cavity. Innumerable bacteria were seen on the films. On the first day the films were obtained from a part of the surface of the wound covered with a dry scab, and contained but a few organisms.

On October 27, one week after the patient's arrival, the wound was cleansed. On October 30 the number of bacteria began to diminish (Fig. 23), but from November 2 to 22 it fluctuated in the neighborhood of twenty per field. After November 22 the wound rapidly became sterile. Closure on November 30 with horse-

hair, but the cutaneous tissues were taut and were not present in sufficient quantities around the fracture. The cavity was filled with chloramine paste and every other day sufficient paste was added to maintain the sterility of the cavity. On December 13 the tissues appeared to have become sufficiently supple again to permit of their being reunited. The site of fracture was filled with an adipose graft and the tissues were completely sutured (Fig. 21).

In order that the technique of applying adipose grafts may be successfully accomplished, a few special precautions should be observed.

The liberation of the edges of the cicatrix must be followed by careful hæmostasis. At this stage the horsehair stitches to be used for the suture are inserted. The center of these threads is placed along

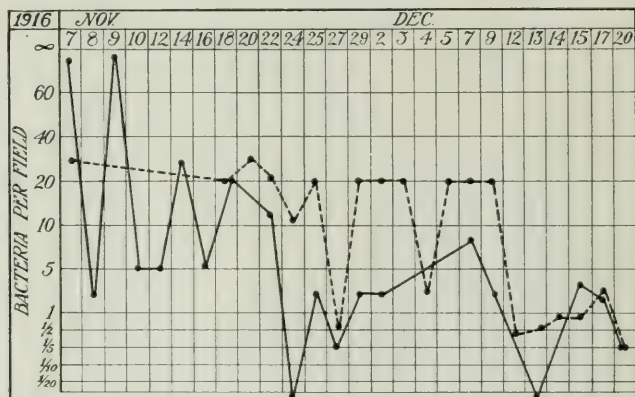


FIG. 14. Bacteriological curve of films from wounds in Case 4.
 —————, anterior wound; - - - - - , posterior wound.

the ends of the wound, in such a manner that the graft can be slipped into the cavity of the fracture without catching in them. The graft tissue is then removed from the subject himself (after both gloves and instruments have been changed), either from the buttock or the antero-external region of the thigh. The patient should not be turned in order to remove the graft, for this may involve faults of asepsis. Before sliding the grafts into the cavity, the periphery of the cutaneous opening should be carefully protected by means of compresses, in such a way that there is no danger of either the skin or horsehair being touched. Once the graft is in place the threads need only be drawn.

It has been seen in Case 6, how difficult it sometimes is to obtain a sufficient amount of skin to cover an old fracture. In certain cases failures result, and, after the wound has been closed as well as possible, the cavity must be allowed to fill up spontaneously. In such cases it is well to preserve its sterility by filling it up every day with chloramine paste.

The following is a good example of this difficulty.



FIG. 15. Combination roentgenogram and drawing of Case 4. The roentgenogram was taken after removal of sequestra and necrosed end of lower fragment of the bone; the drawing showing the operation performed. *M. P.*, medullary plug; *S.*, sequestra.

Case 7.—Open fracture of the leg, caused by a horse's hoof. Infection. Closure of the wound subsequent to operation and sterilization. Insufficiency of cutaneous tissue.

J. M., age 43; wounded August 17, 1916; operated upon in another hospital on August 18. October 8 entered Hospital 21, fifty-one days after being injured.

The first operation was an osteosynthesis, performed with a Lambotte plate which was removed 38 days after its insertion.

Examination.—On the middle third of the leg, bordering on the lower third, there was a cicatrix measuring 20 centimeters in length by 0.5 centimeter in width, the lower part being detached by the distance of 5 centimeters. At this spot, the surface was inflamed and suppurating. Fracture non-consolidated and painful; the limb was cedematous. Films taken from the tissues, stained violet by an antiseptic, showed but few bacteria. The roentgenogram (Fig. 24) showed a transverse fracture of the tibia at the juncture of the middle and lower third. The fracture of the fibula was situated 2 centimeters higher. The fragments of

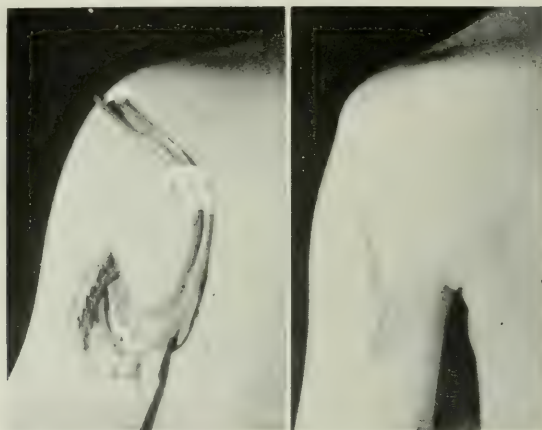


FIG. 16 (at left). Case 5. Wound of upper third of arm and in axillary region.
FIG. 17. Wound sutured.

the tibia were placed end-to-end. Several annular sequestra, corresponding to the screws of the plate of Lambotte, could be distinctly seen in the bone ends. Disinfection with 10 per cent chloramine paste.

Rapid cicatrization, except at the level of three fistulæ leading to a portion of exposed bone. The narrowness of these fistulæ made it impossible to take the secretions for examination.

Operation.—On November 13 the scar and the granulations were excised. The periosteum was ruginated at both ends of the bone, these being connected by means of fibrous tissue, whereupon the circular orifices corresponding to the roentgenogram were disclosed. Each of these openings was surrounded by a dark bony shell, which was cylindrical and mobile. These two bone ends were hollowed

out, and the intermediary fibrous tissue was removed, and by this means a regular drainage canal was obtained, formed by the two bone segments.

Flushing every two hours with Dakin's solution (Fig. 25).

The following evening the temperature was 38°C. The bacteria, which on November 14 were infinite in number, rapidly diminished (Fig. 27). The focus was sterile on November 26. Closure on December 6. The skin although taken



FIG. 18. Combination roentgenogram and drawing, Case 5, showing fracture of the humerus and operation performed. *M. P.*, Medullary plug; *S.*, sequester.

from remote parts, was unable to cover the middle portion of the wound. A section, 5 centimeters in length, at the site of the old fracture, remained open. The bone cavity was filled with chloramine paste. On each succeeding day after the operation the paste was renewed. Complete cicatrization twenty days after closing the wound (Fig. 26).

It is probable that in the case of very old fractures a considerable amount of difficulty would be encountered in covering the operated

surfaces with healthy skin but our investigation has not yet been extended to this point, and was confined to a case eight months old at the time when surgical intervention was applied. In this case it

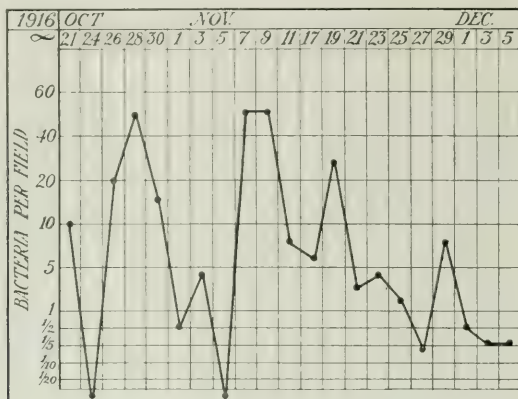


FIG. 19. Bacteriological curve from films of the wound in Case 5.

was found possible to procure a sufficient quantity of autoplasic tissue for the purpose of covering the wound.

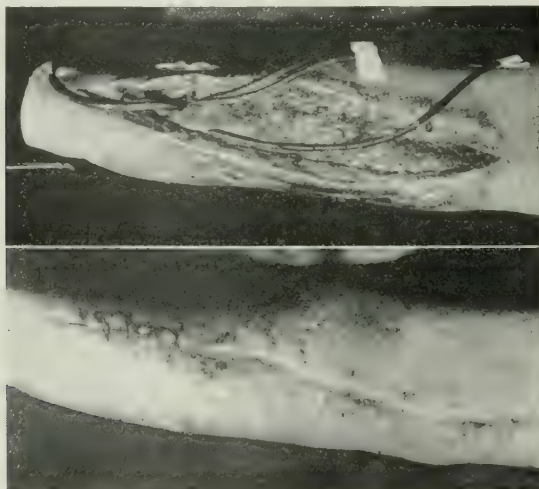


FIG. 20 (at top). Wound of the inner surface of right leg and thigh, Case 6.

FIG. 21. Wound sutured over an adipose graft.

Case 8.—Infected shell fracture of the femur, eight months old. Operation. Sterilization. Closure of wound thirty-six days after operation.

J. A., age 26; wounded March 13, 1916; operated on in a first-line ambulance. Transferred to Hospital 21.



FIG. 22. Roentgenogram showing fracture of right tibia.

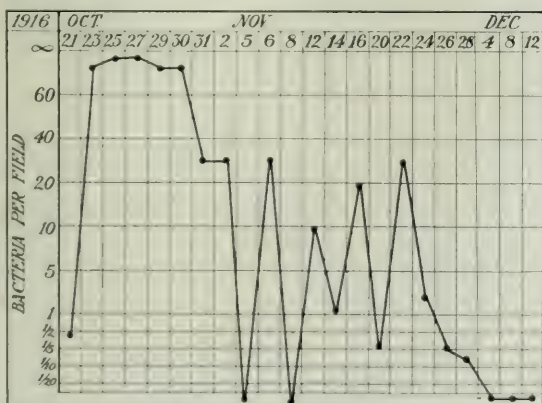


FIG. 23. Bacteriological curve of films from Case 6.

Examination.—November 5. At the juncture of the middle and lower thirds of the right femur there was a large callus. The tissues showed two adherent scars, one antero-internal, the other postero-external. Each of these scars disclosed a fistula through which the denuded bone could be reached with the probe.

The roentgenograms (Figs. 28 and 29) showed a deviation of the axis of the bone. The ends of the bone were covered by periosteal callus. A side view

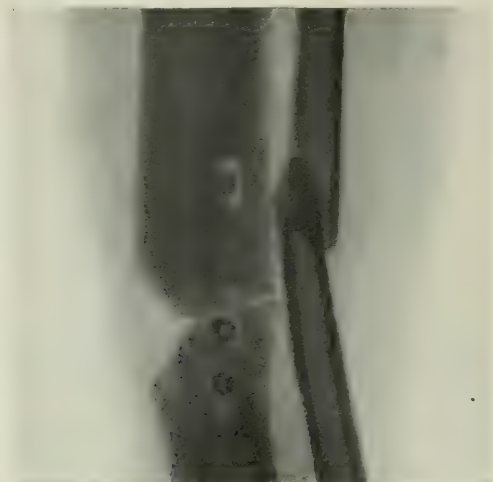


FIG. 24. Roentgenogram showing transverse fracture of tibia and fibula, Case 7.

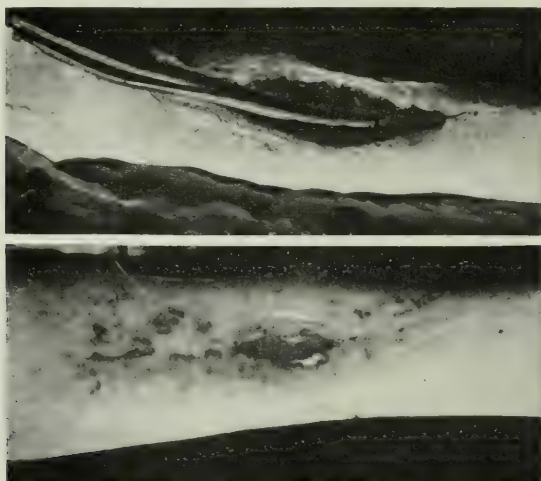


FIG. 25 (at top). Wound of leg, Case 7.

FIG. 26. Wound closed.

showed that between the fragments there was a rarefaction of bone tissue corresponding to osteitis of the callus. In both the callus and the surrounding muscular parts small metallic fragments were discernible.

Both fistulæ contained a large number of bacteria (Fig. 30).

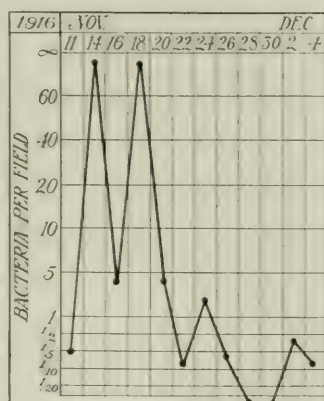


FIG. 27. Bacteriological curve of films in Case 7.

Operation.—November 16. Vertical incision with excision of the walls of the antero-internal fistula. Liberation by means of the rugine of the inner side of the callus. The bony opening of the fistula was enlarged with the gouge and mallet. Next, a funnel-shaped opening was made in the callus by means of the gouge-forceps, until the zone of osteitis was reached, which in the side view of the

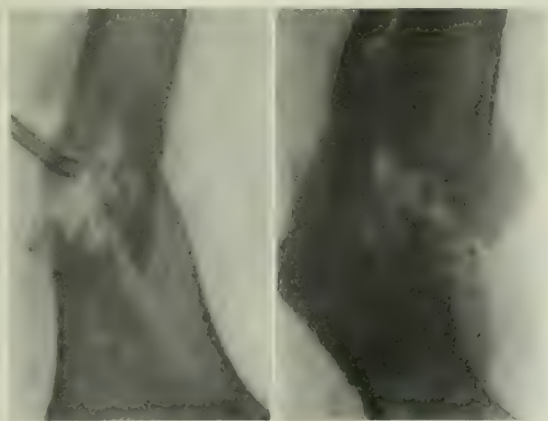


FIG. 28 (at left). Case 8. Roentgenogram showing fracture of femur.

FIG. 29. Case 8. Roentgenogram showing fracture of femur.

roentgenogram appeared as a light spot. This procedure was repeated in the case of the postero-external fistula, and by this means one single and regular bone cavity was formed, terminating in healthy bone and opening widely to both sides of the thigh.

Flushing every two hours with Dakin's solution.

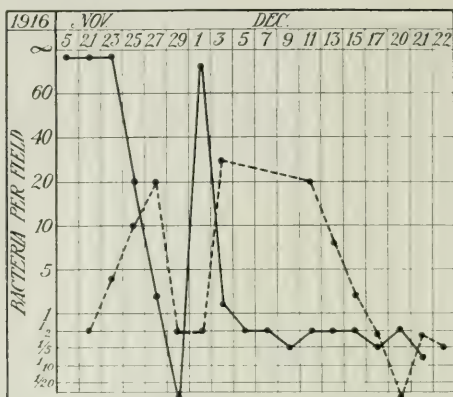


FIG. 30. Bacteriological curves from films in Case 8.



FIG. 31. (at top). Case 8. Antero-internal wound of thigh.

FIG. 32. Case 8. Postero-external wound of thigh, closed.

After December 3, the antero-internal wound (Fig. 31) rapidly became sterile. The postero-external wound was sterile on December 17.

Closure.—On December 22, the postero-external wound had become linear in shape and closed up spontaneously (Fig. 32). The antero-internal wound was finally sutured with horsehair.

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HELIOTROPIC ANIMALS AS PHOTOMETERS ON THE BASIS OF THE VALIDITY OF THE BUNSEN- ROSCOE LAW FOR HELIOTROPIC REACTIONS.

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(Communicated, July 25, 1917.)

While it was customary to express animal instincts in terms of human behavior, one of us many years ago began to replace this anthropomorphic method by the objective and quantitative methods of the physicist. These methods were most easily applicable in the case of those instincts familiar to every layman in which animals were apparently attracted or repelled by light. Loeb¹ was able to express the effect of light in these cases in the following terms: Certain animals are compelled automatically to orient their body in such a way that symmetrical elements of their photosensitive surface are struck by light of the same intensity. In that case the tension and energy production in the symmetrical muscles of both sides of the body are equal and there is no reason for the animal to deviate from this direction of its motion. If, however, the symmetrical photosensitive elements (e.g., the eyes) receive unequal illumination the tension or energy production of the symmetrical muscles is no longer the same and the animal is automatically turned until its orientation is again such that symmetrical photosensitive elements receive the same amount of light.

It was obvious from the observations that this reaction was a function of the constant intensity of light and Loeb assumed that it was a photochemical effect and that the function was probably the law of Bunsen and Roscoe, whereby the effect equals the product $i t$, where i is the intensity of light and t the duration of illumination.²

The proof for the correctness of this view was furnished for the heliotropic curvatures of the hydroid *Eudendrium* by Loeb and Ewald³

and by Loeb and Wasteneys.⁴ The authors could show that if the intensity of light was lowered the time required to call forth the heliotropic curvatures of the polyps had to be increased in such a way as to keep the product it constant.

A second proof was furnished by Ewald⁵ who showed that when constant illumination was replaced by an intermittent one the same effect could only be produced when the product of time of exposure and intensity of intermittent light was the same as that of a constant light. Ewald worked on the orientation of the eye of *Daphnia* to light. This crustacean turns its eye to the light and when the eye is under the influence of two lights of equal intensity the eye is turned in a direction at right angles to the line connecting the two lights. By keeping the one light constant, the other intermittent (through rotating a disk with a sector cut out in front of it) Ewald found that the two lights acted in an equal way when the product it in both cases was equal.

The experiments of *Eudendrium* as well as Ewald's experiments are tedious and it seemed desirable to have a simpler method for the verification of this law. Bradley M. Patten⁶ in working on the heliotropic reactions of the larva of the blowfly (which is negatively heliotropic) determined the path of the animals under the influence of two different sources of light striking the animals simultaneously. Theoretically the animal should creep in such a direction that the intensity of illumination on both sides of its photosensitive elements should be equal, and Patten could prove that for each ratio of the two sources the path was a definite one. By rotating a wheel with a sector cut out before one source of light and cutting down the intensity of the other by a slit Patten could also show that indeed the heliotropic effect is determined by the product of intensity into duration of illumination.

"Using the apparatus described, one of the beams of light was cut down by a diaphragm and the other by an episcotister, so that the light coming from one side was a steady beam of low intensity, and that from the opposite side an intermittent beam in which bright flashes alternated with darkness. The apertures in the sector wheel were adjusted so that the amount of light from each source was equal for a unit time. It has already been established that when the lar-

vae are subjected to equal steady beams of light from opposite directions the aggregate response is almost precisely at right angles to the line connecting the sources of light. The average angular deflection of 200 trails at equality was only 0.09° , when the degrees represented a distance of but 1.5 mm. If the Bunsen-Roscoe law holds for the phototactic response of the larvae, they should orient perpendicularly to the rays of light when subjected to the action of steady and intermittent lights of equal energy per second. The experimental results based on 136 trails made under these conditions show an average angular deflection of but 0.07° from the perpendicular. These results seem to show that in the blowfly larva the phototactic reaction follows the Bunsen-Roscoe energy law."⁷

It seemed desirable to extend the proof for Loeb's theory of animal heliotropism and especially for the validity of the Bunsen-Roscoe energy law to other forms of animals, and we selected for the purpose the reactions of the larvae of the barnacle which were already utilized by Groom and Loeb⁸ in their early experiments on the transformation of positively heliotropic animals into negative ones and vice versa.

These larvae move in a straight line towards or away from a single source of light, and when two lights of equal intensity are given they move in a line at right angles to the line connecting the two lights. These animals are small and can be obtained in large numbers. They were made to collect in the corner of a dish with a little sea water and were then sucked up into a pipette which was blackened with the exception of the opening. When such a pipette is put into a glass dish with parallel walls whose bottom is black (by putting paraffin blackened with lamp black at the bottom of the dish) the larvae will flow out in a fine stream and swim when they are positively heliotropic in a straight line towards the source of light. They thus form a rather narrow white trail on the dark bottom and it is possible to measure the angle of this trail with the line connecting the two lights. In this way in each observation the trail of thousands of individuals is measured. By using one constant and one intermittent source of light and comparing the results with those obtained by two constant lights we can test the validity of the Bunsen-Roscoe law.

The method of the experiments was as follows: *a b c d* (fig. 1) is a square dish of optical glass with blackened bottom and containing a

layer of sea water. *A* and *B* are two lights, the intensity of which is determined by a Lummer-Brodhun contrast photometer. In front of each light is a screen with a round hole permitting a beam of light to go to the dish. The lights and the dish *a b c d* are so adjusted that the two beams of light striking the sides *a b* and *b c* at right angles cross each other in the middle of the dish. The light *A* is fixed while the light *B* is movable on an optical bench. The experiment is made

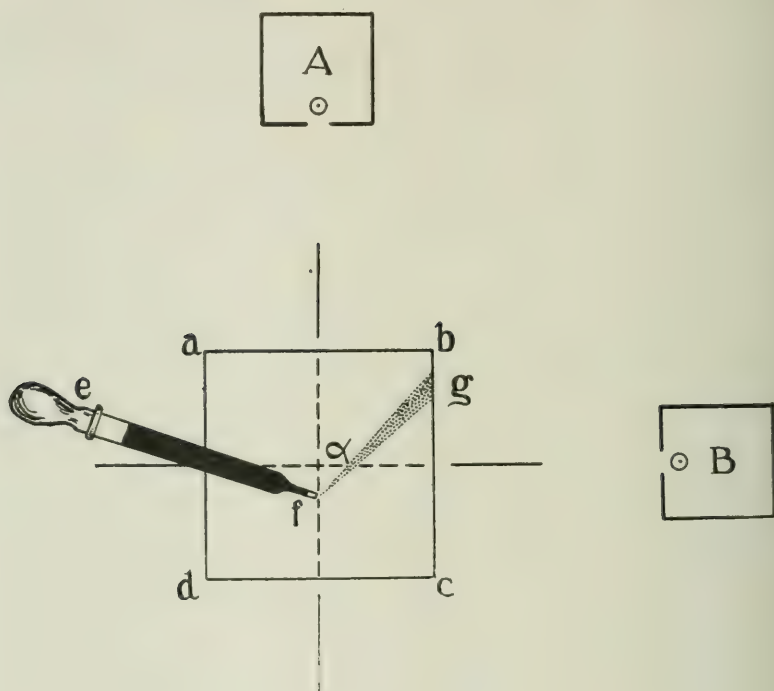


FIG. 1.

in a dark room and the lights *A* and *B* are enclosed in a box. At the beginning of the experiments the pipette is filled with a dense suspension of larvae in sea water and then put with its point touching the bottom of the dish. The animals flow out in a fine stream which is narrow at the opening of the pipette and widens slightly, owing probably to the negative stereotropism of the animals. A glass plate (fig. 2) *h i k l*, which has a strong red line *n o* and a fine parallel

line pg (cut with a diamond) is then put on the dish and so adjusted that pg is in the middle of the stream fg of the animals. Then the angle α which pg makes with the perpendicular from A on ab is measured. This perpendicular is marked in the form of a red line on the black base on which the glass vessel $abcd$ stands. The angle α is measured with a goniometer. When the lights are equal in intensity α should be 45° ; if the two lights have different intensities and if A be the stronger light α should become smaller with increasing difference in intensity. The individual measurements vary comparatively little, as long as the difference in the intensity of the two lights is not too great; for this reason our observations do not go beyond a wider ratio of the two lights than 10:1. Table 1 gives the results. A is always the stronger light. Each table is the average of from 40 to 60 individual observations, each being the average of the path of many thousands of animals.

In the next series of experiments an opaque rotating disk with one sector cut out was placed before light B . In one set of experiments the sector cut out was 90° . The rate of rotation (by an electric motor) was 1,500 to 2,500 revolutions per minute.

The other light was constant and its position was chosen on the assumption of the validity of the Bunsen-Roscoe law for these cases. Thus when the two lights without sector were equal at a given distance of A , by putting the 90° sector before B , it was assumed that the ratio of effects would be the same as if, with constant light, B had been placed at the double distance and the ratio of intensities of the two lights had been 4:1. Going on such a calculation we should expect the same values for α as in Table 1.

As one sees the observed values are slightly smaller but practically identical with the values obtained when the two lights are constant. The deviation is probably due to the fact that the photochemical efficiency of an intermittent light is a trifle less than that calculated on the basis of the Bunsen-Roscoe law.⁹

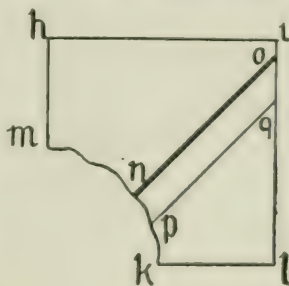


FIG. 2.

TABLE 1.

	Value of α for different ratios of intensities of the two lights.			
Ratio of the two lights.....	1: 1	2: 1	4: 1	10: 1
Value of α (direction of path).....	45.6°	40°	34.4°	28.8°

TABLE 2.

	Value of α when one light is intermittent (90° sector) and the other constant and the efficiency of the two lights is calculated on the basis of the validity of the Bunsen-Roscoe photochemical law.		
Ratio of the two lights.....	1: 1	2: 1	4: 1
Value of α	44.2°	38.3°	34.1°

We carried out some experiments with a sector of 144°. When the efficiency of both lights was equal on the assumption of the validity of the Bunsen-Roscoe law α was found to be 44.9° (instead of 45°), and for the ratio 2:1 α was found to be 38.8°. The values are, within the limits of error, identical with the values in Tables 1 and 2.

SUMMARY.

The paper gives some new quantitative experiments proving that the 'instinctive' motions of animals to light are phenomena of automatic orientation (heliotropism) and a function of the constant intensity of light; the exact expression of the function being the Bunsen-Roscoe law of photochemical action.

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⁴ Loeb, J., and Wasteneys, H., *J. Exp. Zool., Wistar Inst., Philadelphia*, **22**, 1917, (187).

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ON THE INFLUENCE OF FOOD AND TEMPERATURE UPON THE DURATION OF LIFE.

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I. Introductory Remarks.

In order to find out the nature of the causes which determine the natural duration of life of metazoa a quantitative method is required, which permits us to represent the duration of life as the numerical function of one variable. Starting from the idea that chemical conditions in the organism are one of the main variables in this case, one of us raised the question whether there was a definite temperature coefficient for the duration of life and whether this temperature coefficient was of the order of magnitude of that of a chemical reaction.¹ The first experiments were made on the unfertilized and fertilized eggs of the sea urchin and could only be carried out at the upper temperature limits of the organism, since at ordinary temperatures this organism lives for years. In the upper temperature region the temperature coefficient for the duration of life was very high, probably on account of the fact that at this upper zone of temperature death is determined by a change of the nature of a coagulation or some other destructive process. Moore,² at the suggestion of Loeb, investigated the temperature coefficient for the duration of life for the hydranth of a tubularian at the upper temperature limit and found that it was of the same order of magnitude as that previously found for the sea urchin egg. In order to prove that there is a temperature coefficient for the duration of life throughout the whole scale of temperatures at which an organism can live experiments were required on a form whose duration of life was short enough to measure the duration of

¹ Loeb, J., *Arch. ges. Physiol.*, 1908, cxxiv, 411.

² Moore, A. R., *Arch. Entwicklungsmechn. Organ.*, 1910, xxix, 287.

life even at the lowest temperatures. Organisms especially fit for this purpose are insects.

We selected for this purpose the fruit fly (*Drosophila*) which can easily be raised in large numbers on a suitable culture medium in Erlenmeyer flasks. Since Metchnikoff pointed out that the poisons produced by bacteria in the intestine may shorten the duration of life it was necessary to work with flies whose whole body (the intestine included) was entirely free from microorganisms. We succeeded in producing such cultures of flies free from all microorganisms with the aid of a combination of methods introduced by Bogdanow³ and by Delcourt and Guyénot.⁴ Such flies will be designated as "aseptic." We have already published a preliminary report on some of our experiments⁵ and intend to give in this paper the full results of our investigations.

The results published on aseptic flies, *i.e.*, flies free from microorganisms, in the preliminary paper, were as follows.

1. With a supply of proper and adequate food the duration of the larval stage is an unequivocal function of the temperature at which the larvæ are raised, and the temperature coefficient is of the order of magnitude of that of a chemical reaction, *i.e.*, about 2 or more for a difference of 10°C. It increases at the lower and is less at the higher temperatures.

2. The duration of the pupal stage of the fly is also an unequivocal function of the temperature and the temperature coefficient is for each temperature practically identical with that for the larval stage.

3. The duration of life of the imago is, with proper food, also an unequivocal function of the temperature and the temperature coefficient for the duration of life is within the normal temperature limits approximately identical with that for the duration of the life of the larva and pupa.

From this approximate identity of the temperature coefficients for the three stages of the life of the fly we drew the conclusion that the

³ Bogdanow, A. E., *Arch. Physiol.*, 1908, Suppl., 173.

⁴ Delcourt, A., and Guyénot, E., *Bull. Sc. France et Belg.*, 1911, xlv, 249. Guyénot, *Recherches expérimentales sur la vie aseptique et le développement d'un organisme en fonction du milieu*, Thèse de Paris, 1917.

⁵ Loeb, J., and Northrop, J. H., *Proc. Nat. Acad. Sc.*, 1917, iii, 382.

limiting factor for the duration of each of the three stages is a process affected in the same way by the temperature. If it be true that this terminating factor for the larval or pupal stage is the production of a certain type of substance in sufficient quantity (as suggested by the influence of thyroid substance on the termination of the tadpole stage in the frog) or the removal of an inhibiting substance, it follows that a similar cause may be likely to exist for the termination of the last stage in life or for the duration of life.

In this paper we will discuss more fully the influence of the two main factors determining the duration of life, namely, food supply and temperature.

II. Influence of Different Kinds of Food on the Duration of Life of the Imago.

In some insects the imago takes up no food (as, *e.g.*, in the silk worm) but the duration of the life of the imago of the fruit fly depends on the nature of the food, though in an altogether different way from that of the larva. The growth of the insect takes place in the larval stage while neither the pupa nor the imago grows. It was found that while the larvæ cannot grow on "glucose-agar"⁶ unless yeast is added, the imago can live as well on "glucose-agar" alone as when yeast is added. This difference need not surprise us since the larva needs food containing *all* the building stones required for the synthesis of the compounds of its body while the imago, which does not grow, can live on food which is lacking in certain ("accessory"?) substances found in the yeast, presumably because such accessory substances are no longer needed in the fully grown organism or are needed in such small quantities that they can be supplied by the hydrolytic processes going on in its own cells.

Larvæ were raised on yeast at room temperature and the newly hatched flies were then put immediately after hatching upon different

⁶ "Glucose-agar," which proved an excellent culture medium for the flies in our experiments, had the following usual composition. 1 pound of beef was freed from fat, put into 1 liter of water, and placed in the refrigerator over night; boiled 30 minutes, filtered, 20 gm. of agar were added, and boiled till dissolved; 10 gm. of peptone added and boiled 20 minutes; neutralized to phenolphthalein, boiled 7 minutes, cooled to 60°; 2 eggs added, filtered, 20 gm. of glucose added.

culture media and the duration of life was noticed. All experiments were made with aseptic flies under aseptic conditions in thermostats at 25° and 30° respectively.

It was found (Table I) that on agar alone or on agar with the necessary salts the imagos lived less than 2 days at 25° while if dextrose and salts were added to the agar they lived over 8 days at 25°, and on "glucose-agar" they lived 28.5 days at the same temperature. As the experiment for 30° shows, they lived as long on "glucose-agar" alone as when yeast was added.

TABLE I.
Effect of Food on Duration of Life of the Imago (Both Sexes).

Food.	1 gm. washed agar + 100 cc. H ₂ O.	1 gm. agar 0.1 " K ₂ HPO ₄ 0.1 " MgSO ₄ 100 cc. H ₂ O.	1 gm. agar 0.1 " K ₂ HPO ₄ 0.1 " MgSO ₄ 2.0 " dextrose 100 cc. H ₂ O.	"Glucose-agar."		"Glucose-agar" + 5 gm. yeast per 100 cc.
				a.	b.	
Temperature.....	25°	25°	25°	25°	30°	30°
Average duration of life, days.....	1.92	1.75	8.25	28.5	13.7	13.1

TABLE II.
Effect of Sex (30°, "Glucose-Agar" Food).

Sex.	♂♂ ♀♀	♂♂	♀♀
Duration of life, days.....	13.1	15.7	13.3

In these experiments both sexes were used. It was found that the isolated males lived a little longer than the isolated females or the males when mixed with females (Table II). "Glucose-agar" served as food.

III. Influence of Temperature on the Duration of the Larval Period.

For these experiments the aseptic cultures were kept in water-jacketed incubators regulated to within $\pm 0.1^\circ\text{C}$., and containing water so that the humidity was always about 100 per cent. The cultures of

aseptic larvæ were made in 120 cc. Erlenmeyer flasks containing 10 gm. of yeast, 15 cc. of water, and absorbent cotton. Slight variations in the amount of water, cotton, etc., do not affect the rate of growth. The flasks were sterilized in the autoclave for 30 minutes. Aseptic flies of both sexes were put in and allowed to remain 15 hours at room temperature, during which time a number of eggs were laid. The flies were then removed by the aseptic method devised by Delacourt and Guyénot and the flasks with the eggs put into an incubator. The larvæ hatch in a few hours after the eggs are laid, and at the time the flies were removed from the flask most of the larvæ had already hatched. The duration of the life of the larvæ was reckoned from the time the eggs were placed in the incubator to the time the pupæ were formed. Six to ten cultures were made for each temperature. The figures in Table III are the sums of the number of pupæ forming in the separate flasks on the day noted. In computing the averages for the time required to reach the pupal stage the middle of the interval at which the pupæ were formed was used; *i.e.*, if 48 pupæ formed between the 4th and 5th day they were all considered to have taken 4.5 days to form.

We could not well use temperatures lower than 10° since the pupæ did not hatch at that temperature and it was obvious that the growth of the larva was no longer normal. Temperatures above 27.5° no longer accelerated growth.

If we compute from these values the temperature coefficient Q_{10} for the larval period (Table IV, Q_{10}) we find that it is of the order of magnitude of that of a chemical reaction, namely, 2 or more for a difference of 10°C . The temperature coefficients show, however, the irregularities characteristic of all the temperature coefficients for life phenomena, namely, increasing at the lower limit and diminishing at the higher limit. This peculiarity appears also in ordinary chemical reactions, but to a much less degree; but it appears more approximately to the same extent as in life phenomena in chemical reactions catalyzed by enzymes. It seems more natural to assume, as has been done by Arrhenius and others, that these deviations in the temperature coefficients are due to secondary effects of the temperature (*e.g.*, upon viscosity or the state of aggregation of catalyzer or the injury to the catalyzer or some other variable) than to conclude that

the temperature coefficient does not indicate a chemical (enzyme) reaction.

It is well known that at the upper temperature limits the temperature coefficient of enzyme reactions falls off again when the temper-

TABLE III.

Influence of Temperature on Duration of Larval Period of Drosophila.

Days elapsed after hatching of egg.		Number of pupæ formed at							
Day counted.	Average time.	10°*	15°*	20°*	25°		27.5°**	30.0°*	31.5°**
					a.*	b.**			
1- 2	1.5								
2- 3	2.5								
3- 4	3.5					4	156	93	63
4- 5	4.5				53	29	105	129	333
5- 6	5.5				137	18	12	6	254
6- 7	6.5			47	78				52
7- 8	7.5			65	36				
8- 9	8.5			68					
9-10	9.5			16					
13-14	13.5		2						
15-16	15.5		13						
17-18	17.5		27						
19-20	19.5		14						
21-22	21.5		6						
39-48	43.5	5							
49-58	53.5	20							
59-68	63.5	13							
69-78	73.5	3							
Total number of pupæ		41	62	196	304	51	273	228	702
Average duration of larval period in days (from egg to pupation)		57.0	17.8	7.77	5.82	4.76	4.15	4.12	4.92

* Flies used were of the 20th to 22nd aseptic generation.

** Flies used were of the 29th to 31st aseptic generation.

ature rises beyond a certain point. We were interested to know whether the same was true for the duration of the larval stage of the fruit fly, and found this to be the case (Table IV, flies of the

30th to 32nd aseptic generation). On account of this probably secondary effect of the temperature the curve for the rate of larval development (the reciprocal value of the duration $\frac{100}{\text{time}}$) becomes from 10 to 30° (Fig. 1) almost a straight line; and from 25 to 31.5° (Fig. 2) a curve concave on the lower side similar to that found for many simple enzyme reactions.

TABLE IV.

Effect of Temperature on Rate of Growth of Aseptic Larvæ (Fed on Yeast).

Temperature.	Days required to reach the pupal stage.	Rate $\left(\frac{100}{\text{time}}\right)$	Q ₁₀
	Flies of the 20th to 22nd aseptic generation.		
°C.			
10	57 (pupæ do not emerge).	1.75	10.0
15	17.8	5.62	
20	7.77	12.85	4.0
25	5.82	17.2	
30	4.12	24.25	1.78
	Flies of the 30th to 32nd aseptic generation.		
25	4.76	21.0	1.74
27.5	4.15	24.1	
31.5	4.92	20.3	-1.58

We noticed that the duration of the larval stage for the 20th to 22nd generation of aseptic flies was slightly longer than for the 30th aseptic generation and this was true for all temperatures and for all the stages. We cannot account for this difference at present.

IV. Influence of Temperature on the Duration of the Pupal Stage.

Table V contains the data for determining the influence of temperature on the duration of the pupal stage. The stage terminates with the emergence of the imago from the cocoon. If we determine the

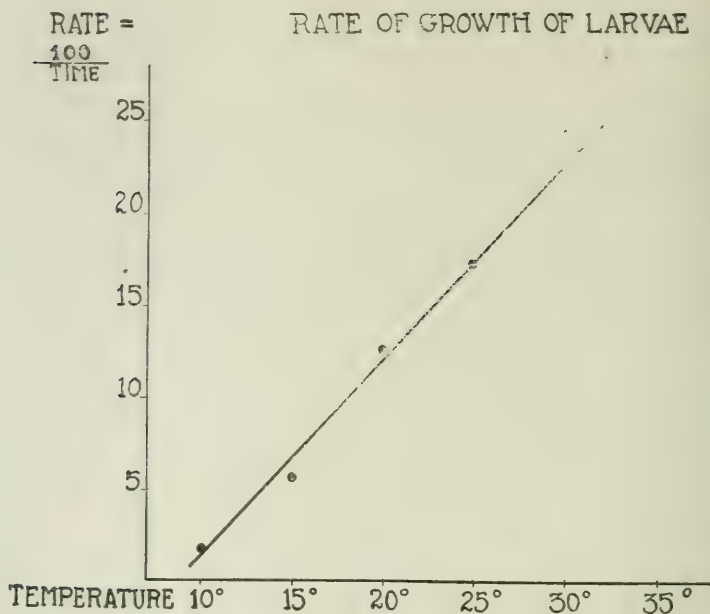


FIG. 1.

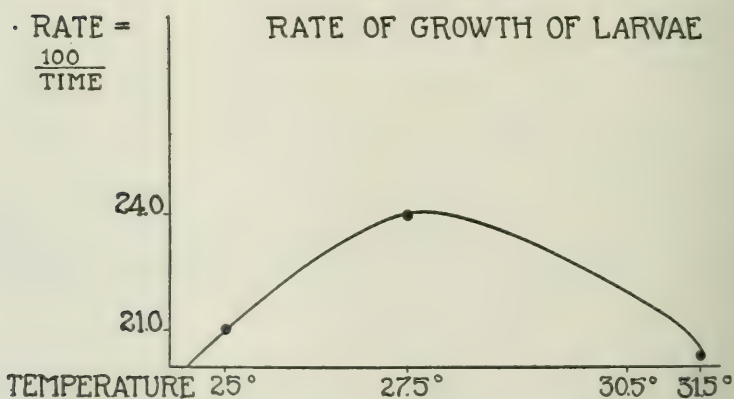


FIG. 2.

time from the laying of the eggs to the emergence of the imago, and deduct from this the time from egg to beginning of pupation (Table III), we get the influence of temperature on the duration of the pupal stage. The temperature coefficient and the rate of development for the different temperatures are given in Table VI. In Fig. 3 the rate curve is plotted which is again between 15 and 30° approximately a straight line.

TABLE V.
Egg-Imago and Pupal Period.

Number of days after larvæ emerge from egg.	Number of imagos which emerge at				
	15°*	20°*	25°*	27.5°**	30°*
1.5					
2.5					
3.5					
4.5					
5.5					
6.5				58	30
7.5				205	114
8.5			6	17	39
9.5			151		
10.5			129		
11.5		4	23		
12.5		21			
13.5		36			
14.5		24			
15.5		21			
16.5		4			
17.5		5			
28.5	9				
30.5	25				
32.5	16				
34.5	10				
36.5	1				
Total number of imagos.	61	115	309	280	183
Number of days egg-imago.	31.5	14.10	10.05	7.35	7.55
Number of days egg-pupae (Table III).	17.8	7.77	5.82	4.15	4.12
Duration of pupa stage.	13.7	6.33	4.23	3.20	3.43

* 20th to 22nd generation.

** 30th to 32nd generation.

V. Influence of Temperature on the Duration of Life of the Imago.

Aseptic larvæ were raised at room temperature (18–20°) on yeast and the flies were removed aseptically from the culture flasks within 15 hours of the time they emerged. They were placed into sterile

TABLE VI.

Temperature and Rate of Development of Pupæ. 20th to 22nd Aseptic Generation.

Temperature.	Days as pupæ.	Rate ($\frac{100}{\text{time}}$)	Q ₁₀
°C.			
15	13.7	7.2	5.0
20	6.33	15.8	
25	4.23	23.7	2.24
30	3.43	29.15	1.53

RATE OF DEVELOPMENT OF PUPÆ

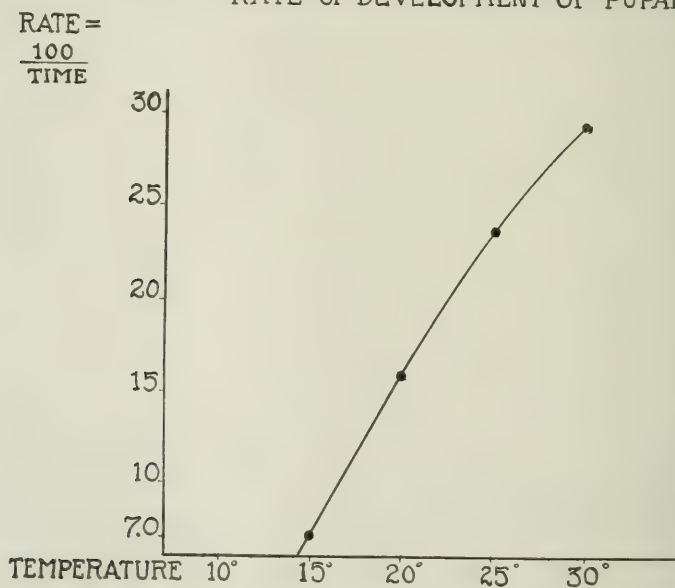


FIG. 3.

500 cc. Erlenmeyer flasks containing 25 cc. "glucose-agar." This food is, as stated, perfectly adequate for the flies but is inadequate for the larvæ which cannot develop on it into flies. This was an important point in the method of our experiments. In order to determine the duration of the life of the imago for a given temperature a

TABLE VII.

Duration of Life of Imago on "Glucose-Agar."

10°		15°		20°		25°		30°	
Days.	No. of dead.	Days.	No. of dead.	Days.	No. of dead.	Days.	No. of dead.	Days.	No. of dead.
17.5	1		1	14.5	1	12.5	6	3	31
24.5	2		4	24.5	5	22.5	30	8	54
31.5	1		0	34.5	14	32.5	20	13	66
52.5	1	38.5	1	44.5	23	42.5	14	18	20
59.5	5	45.5	2	54.5	6			23	43
66.5	1	52.5	3					28	14
73.5	3	59.5	3						
80.5	0	66.5	12						
87.5	2	73.5	18						
94.5	2	80.5	20						
101.5	5	87.5	12						
108.5	2	94.5	11						
115.5	5	101.5	3						
122.5	17	108.5	8						
129.5	14	115.5	4						
136.5	12	122.5	27						
143.5	19	129.5	7						
150.5	7	136.5	7						
157.5	5								
164.5	1								
Total number of flies..	105		143		49		70		228
Average duration of life, days.....	120.5		92.4		40.2		28.5		13.6

definite number of flies were put into a flask, and the number of those which died was ascertained for each day. These flies laid eggs. If the larvæ hatching from these eggs had been able to develop into new flies it would have become impossible to find out the exact death rate of the old flies, since constantly new flies would have been added. This source of error was avoided by using "glucose-agar" as a culture

medium for the old flies on which the eggs laid by the flies during the experiment could not reach the imago stage. By special tests (smears and cultures from dead flies) it was ascertained that all the cultures used in the experiments remained sterile to the end of the experiment. The flasks containing the flies were plugged with cotton. Sufficient food and oxygen were present since cultures containing 30 to 40 flies had the same average duration of life as those containing 10 to 15.

TABLE VIII.

Duration of Life of Imago on "Glucose-Agar."

27.5°		31.5°		33.1°		35°		37.4°	
Days.	No. of dead.	Days.	No. of dead.	Days.	No. of dead.	Days.	No. of dead.	Minutes.	No. dead per 100.
2	10	3	21	1.5	48	0.5	61	10	9
4	13	5	22	2.5	40	1.16	27	30	37
6	14	7	43	3.5	17	1.66	27	50	25
8	10	9	40	4.5	9	2.16	2	70	26
10	14	11	7	5.5	1			90	4
12	9								
14	15								
16	28								
18	6								
20	1								
22	3								
24	3								
Total number of flies..	126		133		115		117		101
Average duration of life, days.....	11.1		6.87		2.41		0.95		0.032

Five to ten separate cultures were used at each temperature, each containing 5 to 20 flies. Several cultures with relatively few flies in each were used since it was more convenient to count the number of dead flies under these conditions. The time was reckoned in the same way as for the larval and pupal periods.

Table VII gives the statistical results of the duration of life of the imago on "glucose-agar." The flies used were of the 20th to 22nd aseptic generation. Table VIII gives the duration for the upper limit of temperatures, 27.5–37.4°. Both sexes were used indiscrimi-

nately in these experiments. In Table IX the average results are tabulated and the temperature coefficients for 10° are given. A temperature of 10° or lower is harmful for the organism, as are temperatures above 30° . In Table IX the temperature coefficients of the duration of life of the imago are computed and the reciprocal value of the duration of life—the rate at which an animal “gets through” with life—is calculated. If we plot this curve (Fig. 4) which corresponds to the rate curve of the larval and pupal stage we find that in the case of the imago it is no longer a straight line but more what we should expect for a chemical reaction curve. The reason that Curve

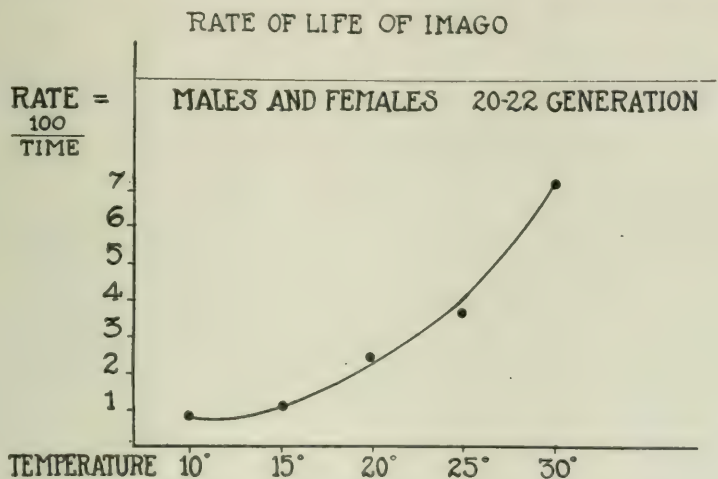


FIG. 4.

4 corresponds more to a chemical reaction curve than Curves 1 and 3 is that the rate does not decrease at the higher temperature limits. This decrease is then a merely secondary phenomenon but it is responsible for transforming the rate curve into a straight line; all of which corroborates our previous statement that the straight line character of the curve does not militate against the assumption that we are dealing in all three curves with a temperature coefficient of the order of that of a chemical reaction.

TABLE IX.

Temperature Coefficient of Duration of Life of Imagos. Males and Females Fed on "Glucose-Agar."

Temperature.	Duration of life of imago.	Rate ($\frac{100}{\text{time}}$)	Q ₁₀
	20th to 22nd generation.		
°C.	days		
10	120.5	0.83	1.70 5.25 1.99 4.4
15	92.4	1.08	
20	40.2	2.49	
25	28.5	3.51	
30	13.6	7.35	
	30th to 32nd generation.		
27.5	11.1	9.00	3.3 630 137 10 ⁶
31.5	6.87	14.55	
33.1	2.41	41.50	
35	0.95	105.2	
37.5	0.032	3,125.0	

In conclusion we will give in Table X the total duration of life and its temperature coefficients for the temperatures at which the animal can complete its cycle.

VI. The Mortality Curve.

When we plot the number of flies which die during successive days in terms of percentage of the original number of flies we get that curve of the death rate usually given in life insurance statistics, namely a probability curve, the ascending branch of which is a little steeper than the descending branch. The death rate of a population of aseptic male flies on "glucose-agar" at 30° is thus given in Fig. 5 and Table XI.

TABLE X.
Total Duration of Life.

Temperature.	Total duration of life.	Rate ($\frac{100}{\text{time}}$)	Q ₁₀
°C.	days		
15	123.9	0.81	5.0 3.0 3.0
20	54.3	1.84	
25	38.5	2.67	
30	21.15	4.65	

Miss Chick⁷ has stated that bacteria are killed by disinfectants at a rate corresponding to that of a monomolecular chemical reaction, *i.e.*, that in each interval of time the same percentage of individuals alive at this time is killed. She was probably led to such an assumption by the fact that the ascending branch of the mortality curve in her experiments was generally very steep. The agencies used by her for killing the bacteria were so powerful that the ascending branch became almost a vertical line, thus escaping detection. Hence she noticed usually only the less steep descending branch which could be interpreted as a monomolecular curve for the reason that her experiments lasted only a short time. In Fig. 6 we give the frequency curve of deaths of a culture of males for a very high temperature, namely,

TABLE XI.
Rate of Death at 30°. Males.

Time.	No. of dead.	No. of dead in interval.	Percentage of original number dying in interval.
days			
3.5	5	5	4.8
7.5	19	14	13.5
13.0	60	41	39.4
18	78	18	17.2
23	93	15	14.4
28	104	11	10.6

⁷ Chick, H., *J. Hyg.*, 1910, x, 237.

39.45°. The ascending branch of the curve is steeper than that for the lower temperature in Fig. 5, but the fact that we are dealing with a probability curve is still very clear. Table XII gives the observations on which this curve is based.

The fact that the frequency curve of deaths is that of a probability curve shows that the difference in the duration of life of different indi-

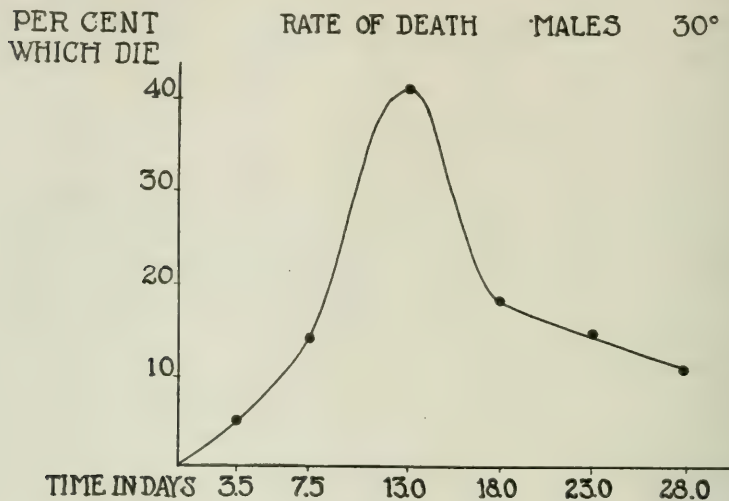


FIG. 5.

viduals for the same temperature is due to individual variation. Incidentally it may be stated that observations on the rate of death of *Fundulus* embryos under the influence of acids, alkalis, and potassium salts show that the mortality curve in these cases is also a probability curve, the descending branch of which is less steep than the ascending branch. This difference may possibly be ascribed to a slight adaptational effect of the destructive agency.

30 to 40 flies were put in test-tubes and placed in a water bath kept at $39.45^\circ \pm 0.02^\circ$, then taken out at time stated and left at room temperature over night. The number of live and dead males was counted after 14 hours.

TABLE XII.

Rate of Death at 39.45° . Males.

Time.	No. of dead.	Total per cent dead.	Per cent dying in interval.
<i>min.</i>			
25	30	4.5	4.5
30	130	17.0	12.5
35	177	34.6	17.6
40	318	54.6	20.0
45	489	72.0	17.4
50	550	82.9	10.9
55	440	88.2	5.3
60	364	91.2	3.0
65	532	97.5	(5.3)
70	350	98.9	1.4
75	609	99.7	0.8

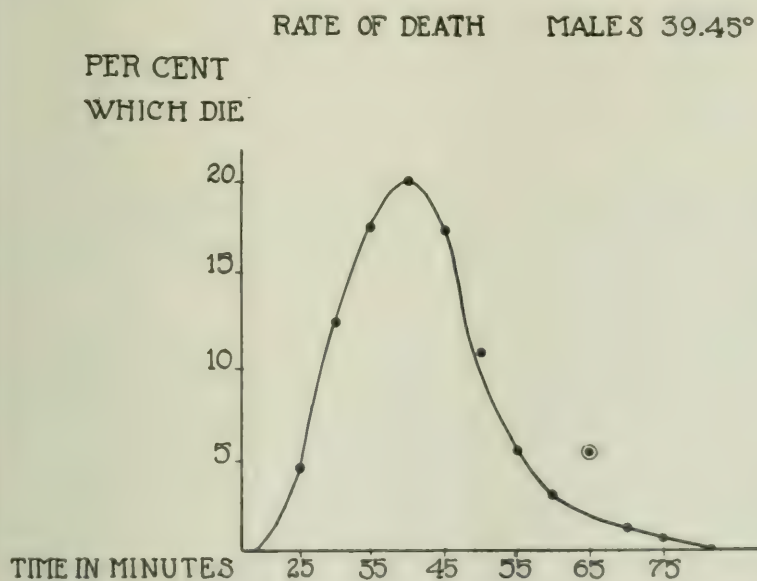


FIG. 6.

VII. Comparison of the Temperature Coefficients for the Larval and Pupal Stage with That for the Duration of Life.

The life of *Drosophila* is normal only within temperatures above 10° and below 30° or roughly between 15° and 25°C. At 10° the larvæ reach the pupal stage but the animals then die without ever emerging from the cocoon. The imago, however, lives at 10°C. At 5° or less the duration of life of the imago is less than a week while at 15° it is 92.4 days. The fact that at 5° the duration of life is less than at 15°C, shows that the former temperature is incompatible with the life of the organism. At temperatures between 27.5° and 31.5°, at which temperature the coefficient for the rate of growth of the larvæ becomes negative, life is also no longer normal. The life of the fly is normal between 15° and 25°C., and it is, therefore, for this range

TABLE XIII.

Temperature Coefficients of Various Stages of Development.

Temperature. °C.	Q ₁₀ for rate of		
	Larvæ.	Pupæ.	Imagos.
15-20	4.0	5.0	5.25
20-25	1.78	2.24	1.99

that a comparison of the temperature coefficients for the three stages becomes permissible. Table XIII shows the temperature coefficients for 15-20° and 20-25° and it is obvious that they are approximately the same for all three stages.

As we have already stated in a previous paper, this proximity of the three values suggests a proximity of the cause limiting the three stages. If the limiting factor for the larval and pupal stages be the production or destruction of a substance ("hormone") the same limiting factor may be suspected for the duration of life. Experiments made by Northrop⁸ show that thyroid has no influence on metamorphosis in the fly.

⁸ Northrop, J. H., *J. Biol. Chem.*, 1917, xxx, 181.

SUMMARY.

1. The paper proves the existence of a definite temperature coefficient for the duration of life of the fruit fly (*Drosophila*).

2. Since the experiments were made with fruit flies free from micro-organisms death cannot be ascribed to bacterial poisoning.

3. The temperature coefficient for the duration of life of the fruit fly is approximately identical with the temperature coefficients for the duration of the larval and pupal stage between 15° and 25°C., i.e., within the limits where development is normal.

4. The duration of the three stages in the life of aseptic *Drosophila*, and the total duration of life is, for temperatures between 10° and 30°, as follows.

Temperature.	Duration (in days) of			
	Larval stage.	Pupal stage.	Life of imago.	Total duration of life from egg to death.
°C.				
10	57	Pupæ die.	120.5	177.5 + X
15	17.8	13.7	92.4	123.9
20	7.77	6.33	40.2	54.3
25	5.82	4.23	28.5	38.5
27.5	(4.15)	3.20		
30	4.12	3.43	13.6	21.15

5. Small variations in the duration of life were noticed in different aseptic generations of the flies; in the 32nd generation the rates were all slightly quicker than in the 20th to 22nd generation.

6. Aside from the temperature the nature of food influences the duration of life and an "adequate" food supply is presupposed for work on the influence of temperature as stated in the previous paragraphs. An adequate food supply for the growing larva includes yeast, while for the adult fly which no longer grows "glucose-agar" (with or without yeast) is sufficient.

7. The observations on the temperature coefficient for the duration of life suggest that this duration is determined by the production of a substance leading to old age and natural death or by the destruction of a substance or substances which normally prevent old age and natural death.

THE EFFECT OF PROLONGATION OF THE PERIOD OF GROWTH ON THE TOTAL DURATION OF LIFE.

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(Received for publication, September 4, 1917.)

It is a well known fact that the growth of an organism may be more or less retarded by supplying it with food insufficient either in quantity or quality. It has been shown¹ that in the case of "aseptic" *Drosophila* the growth of the larvæ may be greatly retarded by growing them on sterile fruit or other substances containing no yeast. The deficiency in this case is probably of the nature of the "vitamines" or growth-promoting substances which have been so thoroughly investigated by Funk, McCollum, Osborne and Mendel, and others. It has also been shown¹ that this prolongation of the larval period is without effect on the duration of the pupal period. It seemed of interest to determine whether the duration of the next stage—the imago—was also independent of the time required to reach that stage. The present experiments were undertaken in order to determine this point. Indirect evidence on this point has been brought forward by Osborne, Mendel, and Ferry,² who have shown that the menopause in female rats is delayed by stunting. Unfortunately their animals died from infection so that the normal duration of life was not determined.

In all these experiments the cultures of flies were, as stated, completely free from microorganisms and the experiments were made in sterile culture media. The Erlenmeyer flasks containing the flies were previously sterilized in the autoclave for 30 minutes as was also the banana or yeast used. The transferring of the sterile flies into a new flask or their removal from the flask was done by the device

¹ Northrop, J. H., *J. Biol. Chem.*, 1917, xxx, 181.

² Osborne, T. B., Mendel, L. B., and Ferry, E. L., *Science*, 1917, xlv, 294.

given by Delcourt and Guyénot,³ which excludes contamination of the fly by microorganisms.

In the experiments described in Table I the eggs were laid on 10 gm. of sterile banana in 120 cc. Erlenmeyer flasks. The parent flies were left in the flask for 4 days, during which time they laid a considerable number of eggs, and were then removed. After some time, as noted in the table, a sterile suspension of yeast in water was added. Upon emerging from the pupal stage the flies hatched from these eggs were transferred aseptically to 500 cc. flasks containing glucose-agar, on which they live as long as on yeast.⁴ The duration of life was then noted. Each experiment is the combined result of from six to nine different cultures. The flies used were of the thirtieth aseptic generation.

The experiment was made in an incubator at 27.5°C.

Table I shows the following facts. When yeast is added at the beginning of the experiment the average total duration of life is 19.3 days at 27.5°, while when the yeast is added on the 8th day of the larval period the total duration of life is 25.5 days at 27.5°, and if the yeast is added on the 10th day the average total duration of life of the fly is 28.9 days. This increase in the duration of life is wholly due to the lengthening of the egg-larval-pupal period by inadequate food supply to the early stages, since the average duration of life of the imago is, as the table shows, approximately the same, namely, between 10.5 and 11.9 days. The larval period was, therefore, prolonged from 8 to 17 days, *i.e.*, over 100 per cent. When the yeast was added on the 12th day or later the life of the larvæ could no longer be saved and they died in the larval stage without being able to complete their development. It must be remembered that the absolute figures given in Table I apply only to the temperature of 27.5°C., and that they would have to be altered for other temperatures.

³ Delcourt, A., and Guyénot, E., *Bull. Sc. France et Belg.*, 1911, xlv, 249.

⁴ Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.

TABLE I.

Effect of Prolongation of Larval Period on Total Duration of Life. Temperature 27.5°C.

Number of days after removal of flies.	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.
0	1-2 mm. larvæ. <i>Yeast added.</i>	1-2 mm. larvæ.	1-2 mm. larvæ.	1-2 mm. larvæ.	1-2 mm. larvæ.
4	Pupæ form.*	<i>Yeast added.</i>			
8	Flies emerge. Number of flies which die.	Pupæ form.	2-3 mm. larvæ. <i>Yeast added.</i>		
10	10			2-3 mm. larvæ. <i>Yeast added.</i>	
11			Pupæ form.		
12	13	Flies emerge.			Larvæ dead.
13		Number of flies which die.		Pupæ form.	
14	14				
15		5	Flies emerge.		
16	10		Number of flies which die.		
17		22		Flies emerge.	
18	14			Number of flies which die.	
19		35	7		
20	9				
21		44	33		
22	15				
23		33	32	5	
24.5	28		41	2	
25		61			
26	6				
27		50	24	15	
28	1				
29	3	21	19	7	
31		6	21	5	
33	3	6	11	8	

TABLE I—*Concluded.*

Number of days after removal of flies.	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.
36			2	2	
38				1	
Total number of flies.	126	283	190	45	
Average total duration of life.	19.3	23.5	25.5	28.9	
Egg-larval-pupal period.	8.0	12.0	15.0	17.0	
Duration of life of imago.	11.3	11.5	10.5	11.9	

* All pupæ formed within a period of 3 days. The 2nd day of pupation is taken as the average time and is the one given in the table. The same approximation is made for the duration of the pupal period. The exact time could not be ascertained in this case since it was impossible to count the imagos as they emerged owing to the necessity of keeping them sterile.

SUMMARY.

The experiments show that if the larval period is prolonged by inadequate feeding the total duration of life can be prolonged. This simply shows that the relative duration of each of the three stages is independent of that of the other two stages; and this would harmonize with the idea that the duration of each of the three stages is determined by the formation (or by the disappearance) of a definite specific substance, as stated in a previous paper.⁵

⁵ Loeb and Northrop, *Proc. Nat. Acad. Sci.*, 1917, iii, 382.

THE DIFFUSION OF ELECTROLYTES THROUGH THE MEMBRANES OF LIVING CELLS.

V. THE ADDITIVE EFFECT OF SALT AND BASE AND THE ANTAGONISTIC EFFECT OF SALT AND ACID.

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I. Introductory Remarks.

In former papers¹ it has been shown that the washed membranes of the egg of *Fundulus* are impermeable for potassium salts, that the addition of a moderate amount of a second salt accelerates the diffusion, and that an addition of a greater quantity of a second salt inhibits the diffusion of potassium salts into the egg. When alone in solution potassium salt cannot diffuse through the membrane of washed eggs until the potassium salt itself has supplied the "salt effect." It was pointed out that these effects of salt on diffusion were analogous to the effects of salts on globulins, which are insoluble in pure water, soluble in a moderate concentration, and insoluble again in a very high concentration of salt.² This would make it appear as if the diffusion of potassium salts depended on the solution of a certain membrane constituent, with qualities resembling those of a globulin. Potassium was used in these experiments since it causes cessation of the heart beat of the embryo as soon as a certain quantity has diffused into the egg. The cessation of the heart beat was, therefore, a convenient indicator for the diffusion of a certain quantity of potassium into the egg.

It seemed desirable to test whether the analogy between the behavior of globulins and the diffusion of electrolytes through the

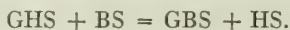
¹ Loeb, J., *J. Bio'. Chem.*, 1916, xxvii, 339, 353, 363; 1916-17, xxviii, 175.

² Loeb *J. Biol. Chem.* 1917, xxxi, 343.

membrane could be carried further, and for this purpose the effect of neutral salts on the diffusion of acids and alkali was examined. Hardy states that there exists an antagonism between the solvent actions of salts and acids while the solvent actions of salt and base are additive. From this difference Hardy has drawn interesting inferences on the manner of combination between globulins, salts, acids, and alkali.

"There is however one feature of fundamental importance which is never obscured, and that is the antagonism between the solvent actions of salts and acids, and the additive nature of the combined solvent action of salts and alkali. This feature arises I believe from the fact that acid globulin is insoluble by salt. Salts will combine with globulin or with alkali globulin to form soluble compounds; they will not so combine with acid globulin.

If this be true, then, when acid globulin is precipitated and redissolved by salt, the acid must be displaced and the globulin redissolved, not as salt-acid globulin but as salt globulin. The displacement of the acid can readily be followed by methyl orange. When HCl globulin is precipitated by a salt this indicator shows that free acid is liberated. But I failed completely to detect the liberation of free alkali when salt is added to a solution of alkali globulin. This suggests that precipitation of acid globulin is partly a definite chemical replacement of acid by salt,



The fact that alkalies slightly assist solution in salts while acids very generally depress it suggests an interesting possibility, namely, that in the compounds GHS and GBS the acid HS and the salt BS are united to the molecule of G in the same way, so that they compete with one another, while in GB and GHS the base and salt are united to different parts of the molecule."³

It was, therefore, expected that if the analogy between the action of electrolytes upon the solution of globulin on the one hand, and the diffusion through the membrane on the other, would hold, that the addition of a salt should increase the rate of diffusion of alkali into the egg and diminish the rate of the diffusion of acid into the egg. This should show itself in this way, that a neutral salt would increase the toxicity of a base and diminish the toxicity of an acid. This is actually the case, as will be shown in this paper. It was necessary to make sure that the salts used were neutral or at least not alkaline, since if they were alkaline the additions of salt to acid would make the acid weaker and when added to alkali increase the alkalinity. This would indeed result in an apparent antagonism of salt to acid and

³ Hardy, W. B., *J. Physiol.*, 1905-06, xxxiii, 323-24.

an apparent additive effect of salt to base. Hence to prove a real antagonism between acid and salt it was necessary to make sure that the salt solutions were not alkaline. Tests were made with phenolphthalein, neutral red, and Congo red. The neutral red tests showed that the salt solutions used (with the exception, of course, of sodium citrate) were all slightly on the acid side of neutrality (all being as red as or more than distilled water). A trace of alkali sufficed to bring them to neutrality. Thus, one drop (0.05 cc.) of N/100 NaOH sufficed to turn 50 cc. of M/4 NaCl or M/32 CaCl₂ or M/32 SrCl₂ from red to yellow (with neutral red as an indicator). Na₂SO₄ was more decidedly acid, 0.5 cc. N/100 NaOH being required to turn 50 cc. M/32 Na₂SO₄ yellow. We are, therefore, dealing in the following experiments with an effect of the neutral salt.

The addition of a neutral salt to an acid with different anion has no effect on the hydrogen ion concentration. Dr. Haas was kind enough to compare with the aid of his indicators the H ion concentration of M/500 acetic acid made up in H₂O and M/32 Na₂SO₄ and found it identical, namely 3.6×10^{-5} .

II. The Antagonistic Character of the Combined Action of Salts and Acid.

Loeb and Wasteneys⁴ have published two papers showing that the adult fish of *Fundulus* can resist acid much better if some neutral salt (NaCl or CaCl₂) is added to the acid. Acid killed these fish by altering the surface of their gills whereby respiration became impossible. The effect of the acid on the external surface of the fish was directly visible inasmuch as the outer lining of the body became white and peeled off. The presence of an adequate amount of salt prevented this effect of the acid when the latter was not too concentrated.

2 years ago the writer published similar experiments on the effect of neutral salt on the rate of diffusion of acid into the egg of *Fundulus*.⁵ The acid had to diffuse through the membrane in order to reach the embryo. As soon as the acid penetrates in sufficient quantity into the egg the embryo begins to coagulate and become white, the thinnest part, the tail, showing this effect earlier than the rest. The heart is protected by the pericardium and therefore the heart beats may still be going on at a time when the tail is already coagulated by the acid.

⁴ Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxiii, 489; 1912, xxxix, 167.

⁵ Loeb, J. *Biol. Chem.*, 1915, xxiii, 139.

As soon as the heart stops beating recovery is no longer possible, since by this time the whole body is coagulated and for this reason the cessation of the heart beat could in this case be used as the criterion for death.

It was found that neutral salts inhibit the rate of diffusion of acid through the membrane. This could be proved by the fact that rhodanates and tartrates prevented the toxic action of acid upon the embryo *inside* the egg, while these salts had no such action upon the fish when outside the egg, the salts themselves being too toxic to be of any use for the fish itself. They were, however, of use in acting upon the external surface of the membrane of the egg, thereby retarding the diffusion of acid through the membrane.

The inhibiting action of salts on the rate of diffusion of acid through the membrane is a function of the anions as well as of the cations. Organic sodium salts antagonize acid better than the inorganic ones and the bivalent anions better than the monovalent ones. Likewise Ca and Sr antagonize better than the univalent cations and also better than Mg and Ba.

The relative efficiency of cations in antagonizing acid is shown in the following table, giving the *minimal* concentration of different neutral salts which permits 50 per cent of the embryos to survive in M/500 acetic acid after 18 hours.

LiCl.....	Die in all concentrations.
NaCl.....	" " " "
RbCl.....	" " " "
CsCl.....	" " " "
MgCl ₂	" " " "
CaCl ₂	M/2048
SrCl ₂	M/512
BaCl ₂	M/32

The superior antagonistic effect of the bivalent over the univalent cations is beyond doubt, as is also the difference between Ca and Sr on the one hand and Mg and Ba on the other.

As far as the anions are concerned, the difference in the antagonistic action between Cl and SO₄ is marked and independent of the nature of the cation. Table I gives the percentage of surviving embryos in M/500 acetic acid in M/8 chlorides and sulfates of different cations.

TABLE I.

After.	Percentage of surviving embryos in $m/500$ acetic acid made up in chlorides and sulfates of different cations.		
	Cation.	Cl	SO ₄
<i>hrs.</i>			
24	Li	0	80
24	Na	0	100
8	NH ₄	0	80
24	Mg	0	100

The sulfates are so much more powerful in their antagonism to acetic acid than the chlorides that at the time when the embryos had all been killed in the chloride solution they were still all active and alive in the sulfate solution.

In order to get a quantitative expression of the relative efficiency of univalent and bivalent anions the embryos were put into $m/500$ acetic acid solution to which various concentrations of different sodium salts were added. That concentration of these salts was ascertained which allowed 50 per cent of the eggs to be alive after 10 hours. This concentration was found to be for

NaCl.....	$m/4$
NaBr.....	$m/4$
NaNO ₃	Slightly $> m/4$
Na ₂ SO ₄	$m/32$

When we add increasing quantities of salt to an acid very soon a concentration is reached where the further addition of salt accelerates the action of the acid; this action takes place at a concentration of the salt where the salt alone is not yet very harmful.

TABLE II.

	Percentage of surviving embryos after 6 hrs. in $m/500$ acetic acid made up in												
	$m/1$	$m/2$	$m/4$	$m/8$	$m/16$	$m/32$	$m/64$	$m/128$	$m/256$	$m/512$	$m/1024$	$m/2048$	$m/4096$
NaCl.....	0	40	100	75	35	5	5	0	5	0	0	0	5
NaBr.....	50	90	95	50	10	0	0	15	5	0	10	5	0
NaNO ₃	15	95	95	40	20	15	5	0	0	10	0	0	5
Na ₂ SO ₄		90	100	100	100	100	80	80	10	5	5	10	10

TABLE III.

	Percentage of surviving embryos after 10 hrs. in m/500 acetic acid made up in												
	m/1	m/2	m/4	m/8	m/16	m/32	m/64	m/128	m/256	m/512	m/1024	m/2048	H ₂ O
NaCl.....	0	10	60	30	0	0	0	0	0	0	0	0	0
NaBr.....	15	85	70	0	0	0	0	0	0	0	0	0	0
NaNO ₃	0	80	40	5	0	0	0	0	0	0	0	0	0
Na ₂ SO ₄		50	100	100	100	90	10	0	0	0	0	0	0

Especially after 10 hours it is clear that the antagonistic effect of M/1 solutions of NaCl, NaBr, and NaNO₃ is less than that of M/2 or M/4 solutions. When the salt concentration exceeds a certain limit its antagonistic action to acid diminishes again.

In a previous paper it was pointed out that when eggs are treated with a low concentration of salt and are *afterwards* exposed to acid the diffusion of acid may be accelerated. Such an effect was never observed when acid and salt were simultaneously in solution no matter how low the concentration of the salt.

III. The Additive Character of the Combined Action of Alkali and Salt.

In working with alkali we have to remember that the CO₂ of the air as well as that produced by the living organism steadily diminishes the concentration of the alkali. This makes it necessary to carry on these experiments in small closed Erlenmeyer flasks in which the volume of solution is large compared with the air space left and the volume of the eggs (20 in each flask). In addition it is necessary to restrict the experiment to short periods. At given intervals the eggs were taken out and examined and the percentage of embryos whose heart was still beating was determined. It was found that the salt always increased the efficiency of the alkali and in no case antagonized it. The following experiments with M/200 NaOH and M/200 tetraethylammoniumhydroxide may serve as an example. The solutions were made up in mixtures of NaCl, KCl, and CaCl₂ in the proportion in which these three salts exist in the sea water; namely, 100 molecules of NaCl, to 2.2 molecules of KCl to 1.75 molecules of CaCl₂ (Table IV).

TABLE IV.

Solution.	Percentage of eggs surviving after 5 hrs. in various alkali solutions made up in NaCl + KCl + CaCl ₂ .									
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512
M/200 NaOH.....	0	0	0	10	30	45	80	100	100	100
M/200 tetraethylammoniumhydroxide.....	0	0	0	0	0	10	10	45	90	100

Even as low a salt concentration as M/32 has an accelerating effect upon the toxic action of alkali. It may be well to point out that these eggs can live for days in neutral solutions of NaCl + KCl + CaCl₂ as concentrated as 2.5 M.

In order to ascertain whether at least a trace of an antagonistic effect of salts upon alkali can be discovered, higher concentrations of the latter were used. The writer did not observe any indication of an antagonistic effect between a strong base and salt while the additive effect was always marked.

The next task consisted in ascertaining the influence of valency and nature of anions and cations of the salt on the additive effect. It was found that the same ions which had the greater antagonistic effects upon acid had the greater additive effect upon bases; *i.e.*, it required less of sulfate than of a chloride to accelerate the toxic effect of a base and less Ca than Na. In order to test this M/400 solutions of ethylamine were used. In a pure M/400 ethylamine solution the embryos live at least for a number of days. If we add, however, a neutral salt the same concentration becomes extremely toxic. If different sodium salts are added the toxicity of the solution increases with the valency of the anion, approximately in harmony with Hardy's exponential law. Table V gives the percentage of surviving embryos in M/400 ethylamine when made up in solutions of NaCl, Na₂SO₄, Na₂ tartrate, Na₃ citrate, CaCl₂, and SrCl₂ after about 22 hours.

Comparing the concentration of these salts which permits 50 per cent of the embryos to remain alive we find this to be for

NaCl.....	M/32
Na ₂ SO ₄	M. 128
Na ₂ tartrate.....	M/128
Na ₃ citrate.....	M/1024

TABLE V.

Solution.	Percentage of surviving embryos after 22 hrs. in M/400 ethylamine made up in various concentrations of salt solutions.									
	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192
NaCl.....	30	50	75	70	75	70	95	90		95
Na ₂ SO ₄	25	30	20	65	80	80	95	95		95
Na ₂ tartrate.....	40	20	25	50	65	80	75			95
Na ₃ citrate.....	0	0	0	10	5	30	70	80		90
CaCl ₂	5	5	0	5	20	30	50	60	85	100
SrCl ₂	25	30	30	35	55	100	85	95	85	90

The ratio of efficiency of monovalent to bivalent to trivalent anion is, therefore, as 1:4:32, while Hardy's rule demands in this case 1:4:16. The exception in citrate is probably due to the fact that citrate is in itself alkaline. In experiments of shorter duration the ratio between tartrate, sulfate, and citrate was M/128:M/128:M/512, which shows citrate to be four times as efficient as sulfate. In the writer's previous papers it was pointed out that Hardy's valency rule holds also for the influence of salts on the rate of diffusion of KCl into the egg.

As far as the cations are concerned, it is obvious that the addition of a slight amount of CaCl₂ accelerates the action of the base considerably more than NaCl, M/1024 CaCl₂ being as efficient as M/32 NaCl. SrCl₂ is slightly less efficient than CaCl₂.

It is of interest that the addition of salts to NH₄OH gave no clear results; there was neither a clear additive effect nor an antagonistic effect between salt and NH₄OH.

IV. Proof That the Salt Action Described in This Paper is Due to an Influence upon the Rate of Diffusion by Alkali and Acid Through the Membrane.

As stated in previous papers the experiments on the *Fundulus* egg have the striking advantage of demonstrating directly whether a salt action is due to an influence upon the rate of diffusion through a membrane or whether it is due to an action on the living protoplasm.

The embryo lives inside the membrane of the egg from which it is separated by a watery liquid. By comparing the effect of a solute on the embryo while *in* the egg, and on the embryo *immediately after hatching*, we can make sure whether or not the effect observed on the egg is merely due to an influence upon the rate of diffusion through the membrane or to an effect upon the embryo inside the egg. When the difference in the two cases is merely one of degree a doubt might still be entertained; but when it becomes one of kind, as is the case in these experiments, all doubt vanishes.

The *Fundulus* embryo inside the egg remains alive indefinitely when the eggs are put into M/400 ethylamine but they all die in less than 24 hours when the M/400 ethylamine is made up in M/64 CaCl_2 or in some other salt. The salt accelerates death. When we make the same experiment on the newly hatched embryo we find that the latter dies in about 20 minutes in M/400 ethylamine and the addition of CaCl_2 does not accelerate the action but retards it markedly, the embryo living from 40 to 60 minutes in M/400 ethylamine made up in M/32 CaCl_2 . The same is true for other salts, *e.g.*, Na_2SO_4 or SrCl_2 ; instead of accelerating the action of alkali on the hatched embryo they retard it. This effect of the salt then is exactly the reverse from the effect of the salt upon the embryo while *inside* the egg. This does not permit of any other interpretation than that the combined effect of salt and alkali upon the egg is due to an action on the membrane surrounding the egg and not upon the embryo inside the egg. We must therefore conclude that the addition of a neutral salt to the alkali allows the latter to diffuse more rapidly into the egg. The embryo which is killed in 20 minutes in M/400 ethylamine, when brought directly into this solution, remains alive in this solution, as long as it is surrounded by the egg membrane, and the reason can only be that this solution never reaches the embryo as long as it is surrounded by the membrane. If, however, a salt of the proper concentration is added to the M/400 ethylamine solution the latter can diffuse into the egg. In a stronger ethylamine solution, *e.g.*, M/200, the embryos are all killed inside of 24 hours when inside the egg. In this case the higher concentration of the alkali itself supplies the "salt effect" upon the diffusion of base into the egg.

As far as the direct action of alkali on the embryo is concerned it is visibly due to the solution of the surface elements of the embryo. In a $M/400$ ethylamine solution the surface of the head is dissolved very quickly, leaving the eyes protruding.

We have already mentioned the fact that tartrates and rhodanates can be used to antagonize the action of acid on the egg, while they cannot be used to counteract the injurious effect of acids on the fish when outside the egg.⁵ This shows that the antagonistic action of salt to acid, as long as the embryo is inside the egg, is due to an effect on the membrane of the egg and not on the embryo itself.

V. Theoretical Remarks.

We have pointed out in this and in a previous paper the analogy which exists between the conditions for the diffusion of electrolytes through the membrane of the *Fundulus* egg and the condition for the solution of globulins. It seems on the whole that those conditions which tend to make globulins soluble also permit or accelerate the diffusion of certain electrolytes through the membrane of the egg of *Fundulus*; while those which render globulins less soluble also inhibit or retard the diffusion of certain electrolytes. Thus it was shown in a former paper that potassium salts diffuse through the membrane of *Fundulus* when the membrane has had a chance to combine with a certain moderate quantity of salt; while either a membrane free from salt or one having an excess of salt is more or less impermeable for the same potassium salt. This corresponds to the fact that globulins are soluble in a moderate amount of salt but insoluble in distilled water or in an excess of salt. It was also pointed out that the valency rule found by Hardy for the precipitation of globulins holds also for the action of salt on the diffusion of potassium through the membrane of the *Fundulus* egg. It was pointed out, however, that there exists merely an analogy and not an identity in the diffusion of salts through the membrane and the solubility of globulins; since the ratio for the solvent and precipitating effect of a neutral salt on globulins differs widely from the ratio for the accelerating and inhibiting effect of the same salt for the diffusion through the membrane.

In this paper the analogy is carried further. Salts and acid have an antagonistic and salt and bases an additive effect upon the solu-

tion of globulins. It could be shown that the relation holds for the diffusion of acid and bases through the membrane. Salts accelerate the diffusion of bases and retard the diffusion of acid through the membrane. The analogy apparently breaks down again through the fact that the increasing valency of both cations and anions of salts increases the antagonistic action to the diffusion of acid and increases the accelerating action on the diffusion of alkali.

Salts, therefore, increase the rate of diffusion of certain electrolytes through the membrane of the egg of *Fundulus* when they are also able to dissolve globulins; and they retard or prevent the diffusion when they are likely to prevent the solution of globulins. The facts, however, do not permit us to say that the substance in the membrane upon which the diffusion of electrolytes depends is a globulin. A difference in the action of salts on acids and bases was also observed by the writer in his old experiments on the swelling of muscle, where he found an antagonistic action of neutral salts to acid but not to base.⁶

Some authors assume that the proof of an increase in permeability of a membrane to *one* substance indicates an increase of permeability to *all* substances. The fact that salts increase the permeability of the *Fundulus* membrane to alkali while they have the opposite effect on the permeability to acids should warn us against such generalizations.

SUMMARY OF RESULTS.

1. When *Fundulus* eggs are put into solutions of bases not sufficiently concentrated to injure the embryo the bases become injurious when neutral salts in low concentration are added. This additive effect between salts and bases was observed also at higher concentrations of the base.

2. When *Fundulus* eggs are put into an acid which kills the embryo rapidly the addition of a neutral salt has an antagonistic effect. The same antagonism between acid and salt was observed also for lower concentrations of acids.

3. The minimal quantity of a salt required for these effects diminishes with an increase in the valency of both anion and cation of the

⁶ Loeb, *Arch. ges. Physiol.*, 1899, lxxv, 303.

salt. In the case of the anion the quantity diminishes according to Hardy's valency rule. In addition the chemical nature of the salt is of importance.

4. It can be shown that the additive effects of salt to base and the antagonistic effect of salt to acid are due in the case of the egg of *Fundulus* to an influence of the salt upon the rate of diffusion of alkali and acid through the membrane of the egg; this influence being accelerating for the diffusion of alkali and retarding for the diffusion of acid.

5. The partial similarity of the action of neutral salts in these cases with the action of salt on the solution of globulins in the presence of acid and alkali is pointed out.

FÉCONDATION ET PHAGOCYTOSE.*

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I.

Le thème de ce travail a été choisi en rapport avec les travaux du grand savant que nous voulons honorer par la présente publication jubilaire. L'idée que la pénétration du spermatozoïde dans l'œuf peut être considérée comme une sorte de phagocytose devrait n'être pas neuve. Mais, dans les sciences de la nature, il est toujours imprudent de se laisser guider par des analogies, au lieu de faits. L'auteur va communiquer, dans ce qui suit, des observations qui gagnent en clarté, si l'on interprète l'absorption du spermatozoïde par l'œuf comme une sorte de phagocytose.

On sait que, d'une manière générale, ce sont seulement les spermatozoïdes de la même espèce qui peuvent pénétrer dans un œuf et non ceux provenant d'espèces différentes. Ainsi l'œuf de l'Oursin peut aisément être fécondé par des spermatozoïdes d'Oursins de la même espèce ou d'espèces voisines, mais non pas—ou seulement d'une façon exceptionnelle—par le sperme de beaucoup d'autres Echinodermes, tels que, par exemple, les Étoiles de mer. Il y a onze ans, l'auteur a trouvé une méthode par laquelle il réussit, à coup sûr, à féconder les œufs de l'Oursin, *Strongylocentrotus purpuratus*, par le sperme de l'Étoile de mer, *Asterias ochracea*, de la côte de Californie. Pour obtenir ce résultat, il faut simplement augmenter l'alcalinité de l'eau de mer. Quand, à 50 cent. cubes d'eau de mer, on ajoute 0.5 c.c. de NaOH à $\frac{n}{10}$, il arrive généralement que tous les œufs d'Oursins, ou presque tous, sont fécondés dans ce liquide, au bout d'une demi-heure, par le sperme de l'Astérie.

* Jubilé E. Metchnikoff. Traduction française de M. Caullery.

Si l'on transporte ensuite ces œufs dans l'eau de mer normale, on constate qu'une partie seulement se développe en larves. Les autres œufs forment la membrane de fécondation; une division nucléaire a lieu, mais ensuite les œufs périssent rapidement. Ces œufs se comportent comme ceux chez lesquels la formation de la membrane de fécondation est provoquée à l'aide du traitement expérimental par un acide gras (ou un autre agent de cytolyse).

Se basant sur ses expériences de parthénogénèse artificielle, l'auteur explique ce fait ainsi: un spermatozoïde a pénétré dans les œufs d'Oursins qui se développent à la suite de la fécondation par le sperme d'Étoile de mer, tandis que ceux de ces œufs, qui forment seulement la membrane de fécondation, puis dégénèrent, sont entrés en contact intime avec le spermatozoïde, sans qu'il y ait eu pénétration complète de celui-ci dans l'œuf. Dans ce cas, les spermatozoïdes fournissent à l'œuf seulement la substance (*lysine*) qui détermine la formation de la membrane, et le développement marche comme s'il y avait eu production artificielle de cette membrane. Mais les spermatozoïdes, dans ces cas, ne pénétrant pas dans l'œuf, n'y abandonnent pas la seconde substance *correctrice*, qui est nécessaire au développement ultérieur, ou plutôt qui empêche la désintégration.

La vérification de cette hypothèse, qui a une grande importance pour la théorie de l'activation, a été entreprise à nouveau l'hiver dernier (1913-1914), par l'auteur, en collaboration avec le D^r Gelarie. On a trouvé qu'en effet le pourcentage des œufs d'Oursins qui, après fécondation par le sperme d'Astérie, se développent en larves, est, dans toutes les expériences, le même que celui des œufs dans lesquels on trouve un noyau spermatique 10 à 20 minutes après la formation de la membrane; tandis que le pourcentage des œufs qui forment seulement la membrane, mais ne se développent pas, est égal à celui des œufs où on ne trouve aucun noyau spermatique.

II.

Ces expériences et d'autres qui ne peuvent être rapportées ici, suggèrent l'idée que l'augmentation de l'alcalinité de l'eau de mer, d'une part rend possible un contact intime du spermatozoïde d'Étoile de mer avec l'œuf d'Oursin, mais qu'elle produit en même temps un

obstacle qui, dans un certain pourcentage des cas, s'oppose à la pénétration complète du spermatozoïde. La phagocytose permet d'obtenir peut-être la meilleure représentation de ce phénomène. Admettons que l'absorption du spermatozoïde dans l'œuf soit, de la part de celui-ci, une sorte de phagocytose; il est clair que l'œuf d'Oursin ne pourra absorber le spermatozoïde d'Astérie qu'en milieu alcalin; ou bien, si on se représente la phagocytose d'après les théories de la tension superficielle, il n'y a étalement, et écoulement du protoplasme ovulaire, autour du spermatozoïde, que si l'alcalinité de l'eau de mer dépasse un peu la normale. Le protoplasma d'œuf non fécondé est entouré d'une gaine gélatineuse, le chorion, que doit traverser le spermatozoïde pour arriver au contact du protoplasma ovulaire. L'auteur a observé que, dans l'eau de mer hyperalcaline, les spermatozoïdes d'Astéries sont agglutinés par cette gaine gélatineuse (et par beaucoup d'autres substances albuminoïdes). Et il est porté à se représenter que, dans une semblable eau de mer hyperalcaline, un certain pourcentage des spermatozoïdes, arrivant jusqu'au protoplasma ovulaire, adhèrent au chorion si fortement, que le protoplasma de l'œuf ne peut pas s'étaler autour de la surface complète du spermatozoïde, mais seulement autour d'une partie de celle-ci. Ces spermatozoïdes sont agglutinés, d'un côté au chorion, de l'autre au cytoplasme ovulaire. Ce dernier s'écoule autour du spermatozoïde autant que possible, mais, comme il ne peut s'étaler entre le chorion et le spermatozoïde (parce que là, ce dernier adhère), il ne se produit pas une pénétration complète du spermatozoïde dans l'œuf. Mais, comme le spermatozoïde est enfoncé en partie dans la couche corticale de l'œuf, ou dans le cône d'attraction, la substance membranogène du spermatozoïde se dissout dans la couche corticale de l'œuf, et la membrane se produit. Cette membrane rend impossible la pénétration de tout autre spermatozoïde dans l'œuf.

L'auteur a récemment trouvé un fait qui concorde avec cette interprétation: le pourcentage des œufs d'Oursin qui, après formation de la membrane, par suite du contact du sperme d'Astérie, se développent en larves, est d'autant plus faible que l'on ajoute plus de NaOH. En élevant la teneur de l'eau de mer en NaOH, on augmente aussi l'adhérence des spermatozoïdes au chorion.

III.

Si ces idées sont correctes, on doit s'attendre à ce que les œufs d'Oursins, dont le chorion est artificiellement éloigné, ne montrent plus ce phénomène singulier. De tels œufs devraient se développer *tous* en larvès, après fécondation par le sperme d'Astérie; car c'était seulement l'agglutination du spermatozoïde au chorion qui empêchait la pénétration du premier dans l'œuf. Herbst a trouvé qu'une faible addition d'acide à l'eau de mer dissout le chorion. Si on place les œufs d'Oursin (*S. purpuratus*), 1 minute $1/2$ à 3 minutes dans 50 cent. cubes d'eau de mer + 3 cent. cubes HCl à $\frac{n}{10}$, le chorion se trouve détruit. Mais, alors qu'il est facile de féconder ces œufs avec du sperme d'Oursin, on ne réussit généralement pas à le faire avec du sperme d'Astérie, même si l'on rend l'eau de mer fortement alcaline.

Cela conduisit l'auteur à chercher des méthodes, par lesquelles il serait possible de féconder avec le sperme d'Astérie, les œufs d'Oursins, même après que le chorion a été détruit par HCl, ou au moins fortement modifié. Dans ce but, l'auteur rechercha si d'autres substances que NaOH ne seraient pas propices à la fécondation et il trouva qu'à cet égard le calcium a une importance vraiment spécifique. Alors que, dans une solution tout à fait dépourvue de calcium, l'œuf d'Oursin ne peut pas être fécondé par le sperme d'Astérie, l'élévation de la teneur en calcium facilite l'hybridation hétérogène jusqu'au double ou au quadruple. L'élévation du taux de calcium rend possible la fécondation dans des solutions où la teneur en NaOH est très basse. Remarquons, entre parenthèses, que, même pour la fécondation de l'ovule d'Oursin par le sperme d'Oursin, le calcium est pratiquement indispensable (il ne peut être remplacé que par le strontium).

Si maintenant on met des œufs d'Oursins normaux dans l'eau de mer hyperalcaline, à laquelle on ajoute en outre du calcium, et si on la féconde par des spermatozoïdes d'Astérie, tous forment la membrane de fécondation, mais le pourcentage des œufs qui donnent des larves est encore plus faible que sans addition de calcium. Dans l'esprit de notre hypothèse, le calcium augmente la tendance du spermatozoïde à rester collé au chorion, ce qui empêche le cône d'attraction d'entourer complètement le spermatozoïde. Mais, si l'on traite les

œufs d'Oursins par HCl, de façon à dissoudre ou à modifier profondément le chorion, il est possible de les féconder par le sperme d'Astérie, dans l'eau de mer hyperalcaline, dont on a élevé la teneur au calcium. On a réussi, dans beaucoup d'expériences, à féconder, par le sperme d'Astérie, 50 à 80 p. 100 de ces œufs, dans ces solutions à teneur augmentée en calcium et hyperalcalinisées. *Pratiquement les œufs se développent tous en larves*, ce qui prouve qu'effectivement c'était le chorion qui empêchait la pénétration des spermatozoïdes dans l'œuf.

Ces observations montrent que le concept de la phagocytose peut faciliter l'interprétation des phénomènes qui conditionnent la pénétration du spermatozoïde dans l'œuf. Il est possible, mais il n'est pas encore prouvé, que l'entrée du spermatozoïde dans l'œuf repose sur des processus de phagocytose (ou de tension superficielle).

THE NEUTRALIZATION OF ANTIPNEUMOCOCCUS IMMUNE BODIES BY INFECTED EXUDATES AND SERA.

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That sterile filtered inflammatory exudates have the power to modify the course of infection was noted long ago. The chief discussion concerning the nature of this phenomenon has been carried on by Bail and his associates and by those who have contraverted their views. Bail¹ gave to the hypothetical substances existing in pathological exudates, which alter the course of infection, the name "aggressins." He thought that they were secreted by the bacteria during their growth in the animal body and acted by inhibiting or by neutralizing the defensive mechanisms of the body. According to this investigator it is to these substances that the bacteria owe their power to invade the body tissues and therefore it is upon their ability to form them that the property of virulence depends.

Wassermann and Citron² have opposed the view of Bail that these so called aggressins represent substances set free by the bacteria during their struggle against the protective agencies of the body, and believe that they represent merely bacterial substances which may go into solution either within the body or during growth or autolysis *in vitro*, and that these substances act by fixing the humoral immune bodies and so rendering them ineffective; that the mode of action of these substances therefore does not differ from that of dead bacteria. It is not german to the present discussion to consider the large amount of evidence which has been brought forward to support the contending views, the chief purpose of the present paper being to record the demonstration of large amounts of substances which neutralize immunity principles in the blood and exudates of animals infected with pneumococci, and to indicate the importance of these substances in the specific therapy of acute lobar pneumonia.

The part which these substances play in experimental pneumococcus infections, especially their effect on phagocytosis, has been studied by Hoke,³ Rose-

¹ Bail, O., *Arch. Hyg.*, 1905, lii, 272.

² Wassermann, A., and Citron, J., *Deutsch. med. Woch.*, 1905, xxxi, 1101.

³ Hoke, E., *Wien. klin. Woch.*, 1905, xviii, 348.

now,⁴ Tschistowich and Jurewich,⁵ Zade,⁶ and Nunokawa.⁷ Tschistowich and Jurewich have made the observation that thoroughly washing virulent, non-phagocytatable pneumococci in salt solution is sufficient to render them phagocytatable. Rosenow, as well as Tschistowich and Jurewich, has also observed that treating non-virulent, phagocytatable pneumococci with the washings from, or extracts of virulent pneumococci is sufficient to render the former non-phagocytatable and therefore virulent, and he has found that this property is retained even after washing in salt solution. This writer has given to the hypothetical substances which may be extracted from virulent pneumococci the name "virulin," and Tschistowich and Jurewich have given to the substances which they have obtained by somewhat similar methods the name "anti-phagin."

It is evident that these observations, especially those of Rosenow, would render necessary an entirely different conception of the phenomenon from that held by Wassermann and Citron.

In a considerable number of experiments, however, I have been unable to confirm the observations of Rosenow that non-virulent pneumococci may absorb and fix something derived from virulent pneumococci which renders the former virulent, and for the present, therefore, I am inclined to accept the explanation offered by Wassermann and Citron, especially since this conception is sufficient to explain all the following observations.

The first observations on which this communication is based were made on the fluid removed from the chests of persons suffering from empyema. The fluid from these cases was examined for its content in pneumococcus immune bodies as tested by agglutination and protection. Similar tests of the patient's blood showed that it possessed well marked protective and agglutinative properties, and we were therefore surprised when we found that the empyema fluid possessed no such powers. A probable explanation seemed to be that, although the immune bodies were originally present in the exudate, they had been absorbed by the bacteria present, just as they may be from immune blood serum when bacteria are added *in vitro*. It occurred to us, however, to test this empyema fluid after removal of bacteria, to determine whether or not there might be present soluble substances,

⁴ Rosenow, E. C., *J. Infect. Dis.*, 1907, iv, 285.

⁵ Tschistowich, N., and Jurewich, Y., *Ann. Inst. Pasteur* 1908, xxii, 611.

⁶ Zade, M., *Z. Immunitätsforsch., Orig.*, 1909, ii, 81.

⁷ Nunokawa, K., *Z. Immunitätsforsch., Orig.*, 1909, iii, 172.

which would fix or divert the immune substances contained in immune serum. The following is a protocol of one experiment.

Case 1.—E. R.; age 19 years. Acute lobar pneumonia followed by empyema due to *Pneumococcus* Type I. 200 cc. of thick pus were removed at operation. A portion of the fluid was centrifugalized at high speed for 30 minutes, the super-

Agglutination Tests						
0.4cc.Serum I + 0.4cc.empyema fluid				30 min at 37° C.	0.1cc.Cult.I	2hrs. 37°-18hrs ice
"	"	(1:10)	"		"	++
"	"	(1:20)	"		"	++
"	"	(1:40)	"		"	+
"	"	(1:50)	"		"	-
"	"	(1:100)	"		"	-
"	"	(1:200)	"		"	-
"	"	(1:10)	NaCl		"	++
"	"	(1:20)	"	"	++	
"	"	(1:40)	"	"	++	
"	"	(1:50)	"	"	++	
"	"	(1:100)	"	"	++	
"	"	(1:200)	"	"	++	
_____ Empyema fluid undiluted					"	-
_____ " 1:20					"	-
_____ " 1:40					"	-
_____ " 1:100					"	-

TEXT-FIG. 1. Protocol of an experiment showing the inhibiting action of empyema fluid on the agglutination of pneumococci by immune serum.

natant fluid was removed to a fresh centrifuge tube and again centrifugalized for 1 hour, and finally diluted with an equal quantity of isotonic saline solution and again centrifugalized for 30 minutes. The perfectly clear fluid as examined microscopically contained no organisms.

This fluid was then tested for its power to cause agglutination of Type I pneumococci and also for its power to inhibit the agglutination of pneumococci by Type I immune serum. The results are given in Text-fig. 1.

The fluid was then tested for its power to inhibit the protective action of immune serum against infection with Type I pneumococci as tested in mice. To guard against the possibility that the fluid, centrifugalized as noted above, might contain an occasional pneumococcus which might interfere with the result, it was heated for 30 minutes at 56°C. Cultures made from this fluid were sterile.⁸ Text-fig. 2 gives the results of this experiment. They show in a striking way that empyema fluids may contain large amounts of soluble substances which inhibit the action of immune serum.

Protection Tests					
Culture dilution cc.	0.2cc. i.h.s. + 0.2cc. empyema fluid	0.2cc. i.h.s. 0.2cc. n.h.s.	0.2cc. NaCl + 0.2cc. empyema fluid	Culture control	Empyema fluid alone cc.
0.1	D. 20 hrs.	S.	D. 30 hrs.	—	0.5 S
0.01	" 21 "	"	" 16 "	—	0.3 "
0.001	" 30 "	"	" 38 "	—	0.2 "
0.0001	—	—	" 30 "	—	0.1 "
0.00001	—	—	" 30 "	D. 30 hrs.	
0.0000001	—	—	" 30 "	" 30 "	

TEXT-FIG. 2. Protocol of an experiment showing the inhibiting action of empyema fluid on the protection of mice by immune serum.

Similar tests have been carried out with a series of these empyema exudates removed by aspiration or at operation. The results have not been so striking in all the cases examined as those shown in the above protocol, though some degree of inhibition has been present in all infected cases. Several sterile serous fluids aspirated from the chest of pneumonia patients, however, have not exhibited this phenomenon. The degree of inhibiting action is apparently dependent upon the degree of infection and the time the infection has lasted before aspiration is performed.

These observations indicate why it is that infections in the partially immunized animal tend to be focal and why, when an animal is infected with organisms of slight virulence, the infection tends to remain localized. It is probable that as soon as bacteria begin to grow in tissue spaces these inhibiting substances appear in the in-

⁸ In making the tests with mixtures of serum and fluid, the mixtures were allowed to incubate for 30 minutes at 37°C. before injection.

flammatory exudate, and when the fluid is not readily absorbed the substances accumulate in large amounts, so that finally, as in empyema, it is practically impossible to produce a focal immunity reaction until the focus is opened and the fluid, with its content of neutralizing substance, is removed by drainage, when the bacteria remaining are no longer protected from the natural or artificial defensive mechanisms of the body and so may be overcome. This conception agrees in the main with the view held by Bail and others, though the application of the theory has previously been made rather to the problems of virulence and infection than to those of recovery. The observations previously mentioned also indicate that favorable results can hardly be expected from the treatment of these focal infections with immune serum, either administered intravenously or injected directly into the focus itself unless the pathological exudate has previously been removed. We have made one attempt to treat a patient suffering from empyema by the direct injection of immune serum into the cavity, but without apparent effect. These observations offer the explanation for the failure. In the treatment of focal infections with immune serum, without drainage, it would be necessary to inject sufficient serum to neutralize all the inhibiting substances present, as well as the amount necessary to prevent the harmful activities of the bacteria themselves.

Our next problem was to discover whether or not the inhibiting substances appear in the blood as a result of septicemia. This was first investigated by inoculating rabbits with very large injections of pneumococcus and testing the blood removed during the height of infection for the presence of these substances. To show that this action is due to soluble substances, and not to the bacteria present, the bacteria have been removed from the serum by filtration before testing. The results of one of these experiments are given in the following protocol.

Rabbit 1.—Weight 1,300 gm. July 11, 1916, 12 noon. Inoculated intraperitoneally with 1 cc. of peritoneal exudate of a rabbit previously infected with Type II pneumococci. 5 p.m. Blood culture shows innumerable numbers of pneumococci.

July 12, 10 a.m. Animal very sick. Blood removed by heart puncture. Serum removed from clot and passed through a Berkefeld filter. Culture of filtered blood sterile. The filtered blood was tested for its power to inhibit the

agglutinating action of immune horse serum, Type II. Table I shows the results obtained.

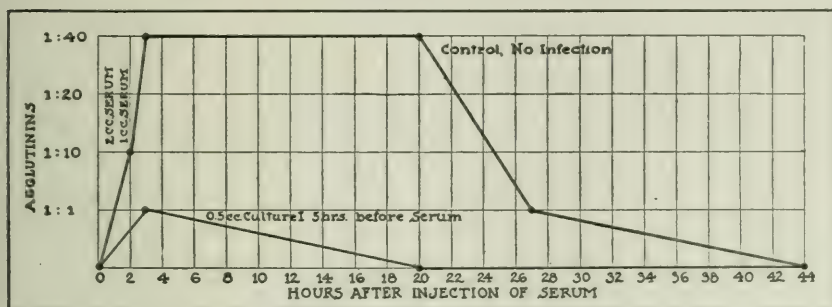
TABLE I.

Agglutination.		Results after 2 hrs. at 37°C. and 18 hrs. on ice.
0.4 cc. of Immune Serum II (1:40) + 0.4 cc. of filtered serum.....		—
0.4 cc. of Immune Serum II (1:50) + 0.4 cc. of filtered serum.....		—
0.4 cc. of Immune Serum II (1:100) + 0.4 cc. of filtered serum.....		—
0.4 cc. of Immune Serum II (1:200) + 0.4 cc. of filtered serum.....		—
	After 30 minutes at 37°C. 0.1 cc. of Culture II was added to each tube.	
0.4 cc. of Immune Serum II (1:40) + 0.4 cc. of salt solution.....		++
0.4 cc. of Immune Serum II (1:50) + 0.4 cc. of salt solution.....		++
0.4 cc. of Immune Serum II (1:100) + 0.4 cc. of salt solution.....		++
0.4 cc. of Immune Serum II (1:200) + 0.4 cc. of salt solution.....		++
0.4 cc. of Immune Serum II (1:400) + 0.4 cc. of salt solution.....		+
0.8 cc. of salt solution.....		0

The results of this and other similar experiments show that specific inhibiting substances such as those which are present in pathological exudates may also be present in the blood when an animal is suffering from a severe septicemia.

Another method which has been used for testing the presence of inhibiting substances in the blood is the following. A rabbit is infected with pneumococci and after the infection has reached its height immune serum is injected intravenously. At the same time, and as a control, a normal rabbit receives the same amount of immune serum intravenously. Within a few minutes and at varying periods following the injection of the serum, samples of blood are removed from both rabbits and tested for their content in antibodies. For this purpose agglutination is employed. If no neutralization of antibodies occurs, it is evident that the content of the blood in agglu-

tinins immediately following the injection should be the same as though the immune serum had been diluted *in vitro* with a quantity of fluid equal to the blood contained in the rabbit, and that by making repeated tests a curve showing the disappearance of the immune bodies by destruction or excretion may be constructed. As a matter of fact, numerous observations in normal rabbits have shown that when the rabbit's blood is tested within a few minutes following the injection of immune serum, its content in agglutinins is about that to be expected when the probable volume of blood in the rabbit and the consequent dilution of the immune serum is calculated. On the other hand, when a similar injection is made into an infected animal,



TEXT-FIG. 3. Curves showing the agglutinating power of the serum of normal and infected rabbits following the injection of immune horse serum.

the agglutinating power of the serum obtained from it is much less than that calculated from the probable dilution; indeed, agglutinating power may be entirely absent. Moreover, when the agglutinating power is present, though lower than that of the serum of the normal rabbit, and curves are made to show the disappearance of the agglutinating power, it is found that the agglutinins disappear much more rapidly from the serum of the infected rabbit than they do from the serum of the uninfected rabbit. Text-fig. 3 shows in a graphic manner the results obtained in one of these experiments.

In these experiments the possibility cannot be excluded that the fixation or neutralization of antibodies is due to the presence of bacteria circulating in the rabbit's blood, but previous observations make it

improbable that the entire phenomenon can be due to this. It seems probable that the neutralization is due to a considerable extent to the presence of soluble inhibiting substances.

This last method of study is directly applicable to patients, and a study of this kind in patients is of importance since it is difficult to produce in animals pneumococcus infections which last over a period of a week or longer, such as those which occur in man. Moreover, it was hoped that this study would offer indications for proper dosage of serum and might even be applicable in the treatment of the individual case. In a series of cases, therefore, the serum has been tested for its content in agglutinating antibodies both before and following the administration of immune serum.

The method of procedure was as follows: Samples of the patient's serum were obtained before any immune serum was administered and also 5 minutes following the first dose. Where more than one dose was administered (and successive doses have usually been given with 6 to 8 hour intervals) other samples were obtained immediately before and 5 minutes following each subsequent dose. Finally, following the last dose, in certain cases, samples were obtained at varying periods to observe the persistence of agglutinins in the blood. The samples from each patient were kept on ice until all had been obtained and they were then tested on the same day and with the same technique for the presence of agglutinins. The agglutination tests were made by the macroscopic method. In each of a series of small test-tubes was placed 0.9 cc. of the serum, or of the diluted serum. To each of the tubes was then added 0.1 cc. of an 18 hour broth culture of pneumococcus of the type to which the infection was due and corresponding to the serum which had been injected. The results were read by transmitted light after 2 hours at 37°C. and again after the tubes had remained on ice over night.

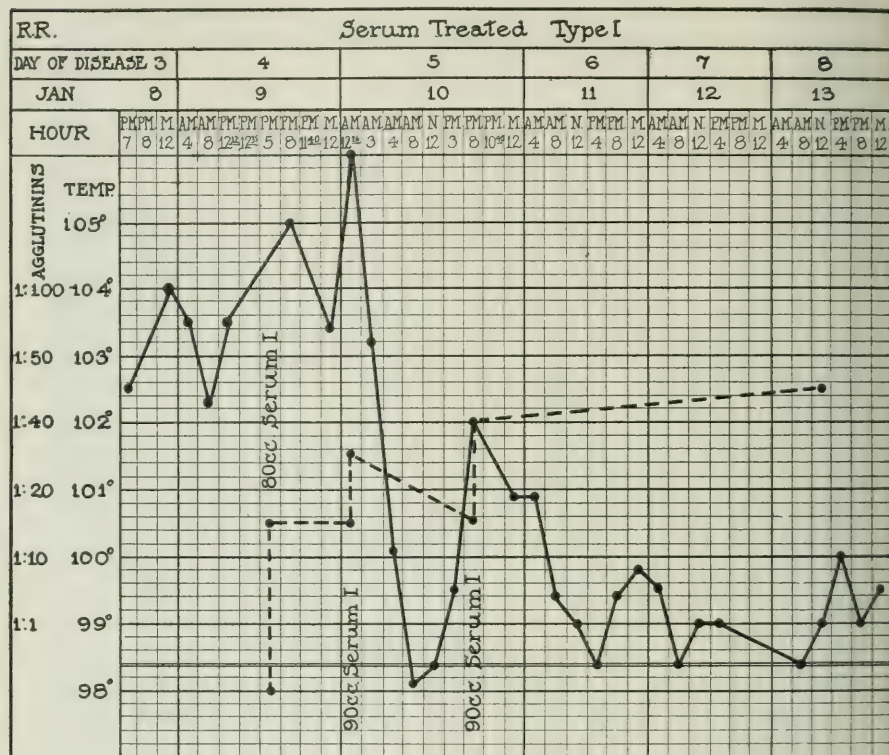
Employing these methods, agglutinin curves have been made from 30 cases suffering from Type I infection and receiving serum, 9 cases suffering from Type II infection, and also serum treated, 7 cases with Type II infection, who received no serum, 4 of Type III, not serum treated, 2 of Type IV not serum treated, and 1 case due to Type IV infection who, through a mistake in diagnosis, received several doses of Type I serum, making a total of 53 cases.

Cases Due to Type I Infection Treated with Serum.

Of these cases, 3 were treated on the 2nd day, 5 on the 3rd day, 5 on the 4th day, 4 on the 5th day, 9 on the 6th day, 3 on the 7th day, and 1 on the 9th day. All but two of the cases recovered. The charts and protocols of several cases, which illustrate the relation of agglutinin titer to the clinical course and to the temperature curve, are given below.

Case 1.—R. R., student; age 22 years. This patient was admitted January 8, 1917 at 7 p.m. suffering from pneumonia involving the left lower lobe. The onset had been quite typical with chill, 48 hours before admission. He was moderately sick; temperature 102.5°F., pulse 115, respirations 30. The leukocytes numbered 31,000 and the blood culture was positive, the plates showing one colony per cc. of blood. The sputum was bloody; a small amount was at once inoculated into a mouse. The following morning tests made of the growth in the peritoneal cavity of the mouse showed that the patient was suffering from an infection with Type I pneumococci. 12.17 p.m. The intravenous injection of antipneumococcus serum was commenced. Although the serum was given slowly, after he had received about 35 cc. he had some signs of serum intoxication, suffusion of the face, respiratory difficulty, and he vomited several times. The administration of serum was therefore at once discontinued. No tests were made of the agglutinating power of his serum before or after this treatment. The patient's condition did not materially change during the afternoon and at 5 p.m. serum was again administered; this time 80 cc. were given without any untoward symptoms. A sample of blood was taken just before and another one 5 minutes following the administration of the serum. When tested later, it was found that the blood before administering the serum contained no agglutinins for pneumococcus; the sample of blood taken following the administration of serum agglutinated Type I pneumococcus in a dilution of 1:15. This represents a concentration of antibodies fully equal to that which might be expected, taking into consideration the titer of the serum injected and the probable volume of the patient's blood. The patient's condition did not materially improve after this injection, so that another dose of 90 cc. of serum was administered at 12 midnight. A specimen of blood which was obtained just before this injection showed that the agglutinating power had not diminished during the time intervening since the preceding dose, and the specimen of blood taken 5 minutes after the serum was injected showed an increased concentration of agglutinins, so that now agglutination occurred with a 1:30 dilution of serum. Immediately following this injection the temperature rose to 106°F. and he had a shaking chill. The temperature then began to fall, being only 98.1°F. at 8 a.m. With this fall in temperature, the patient's condition markedly improved. During the day, the temperature again rose slowly, without, however, any other unfavorable features. As the temperature at 8 p.m. was

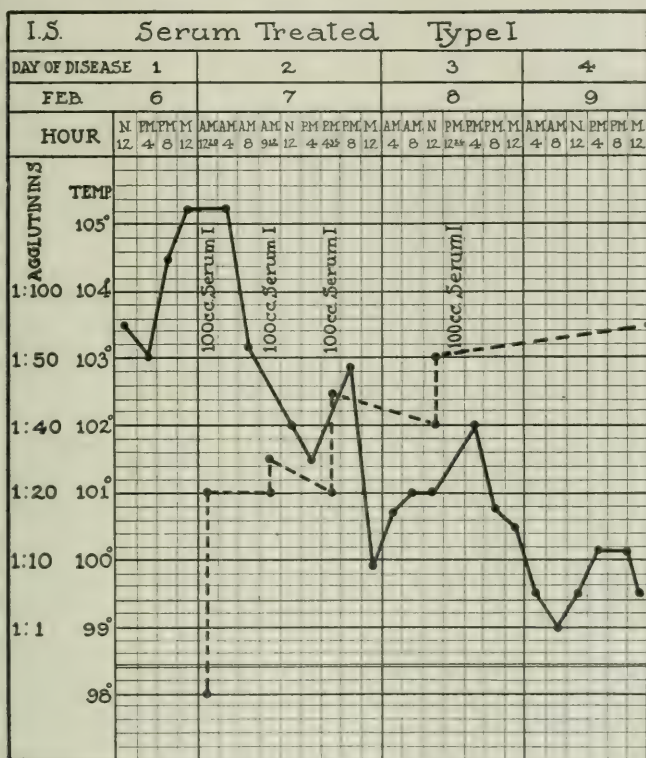
102°F., it was decided to administer another dose of serum, and 90 cc. were given, without any reaction. The test of the patient's serum obtained before this treatment showed that the agglutinins had fallen slightly, agglutination occurring in a dilution of 1:15, but after the treatment the titer again rose to 1:40. Following this treatment the patient made a good recovery.



TEXT-FIG. 4. Chart showing the curve of the agglutinin titer and the temperature curve of R. R.

In this case, therefore, which was mild or of only moderate severity, treated early in the disease, the administration of immune serum was followed by a satisfactory concentration of antibodies in the blood and there was no evidence of fixation or neutralization of the injected immune substances. This is graphically shown in Text-fig. 4.

Case 2.—I. S., tailor; age 32 years. This patient was admitted about 8 hours following the initial chill. There were definite signs of involvement of both lower lobes and he presented all the characteristic features of acute lobar pneumonia. His temperature on admission was 103.5°F., pulse 120, respirations 48. He appeared seriously sick; the blood culture taken on admission was positive. The diagnosis of the type of infecting organism was made by inoculation of sputum



TEXT-FIG. 5. Chart showing the curve of the agglutinin titer and the temperature curve of I. S.

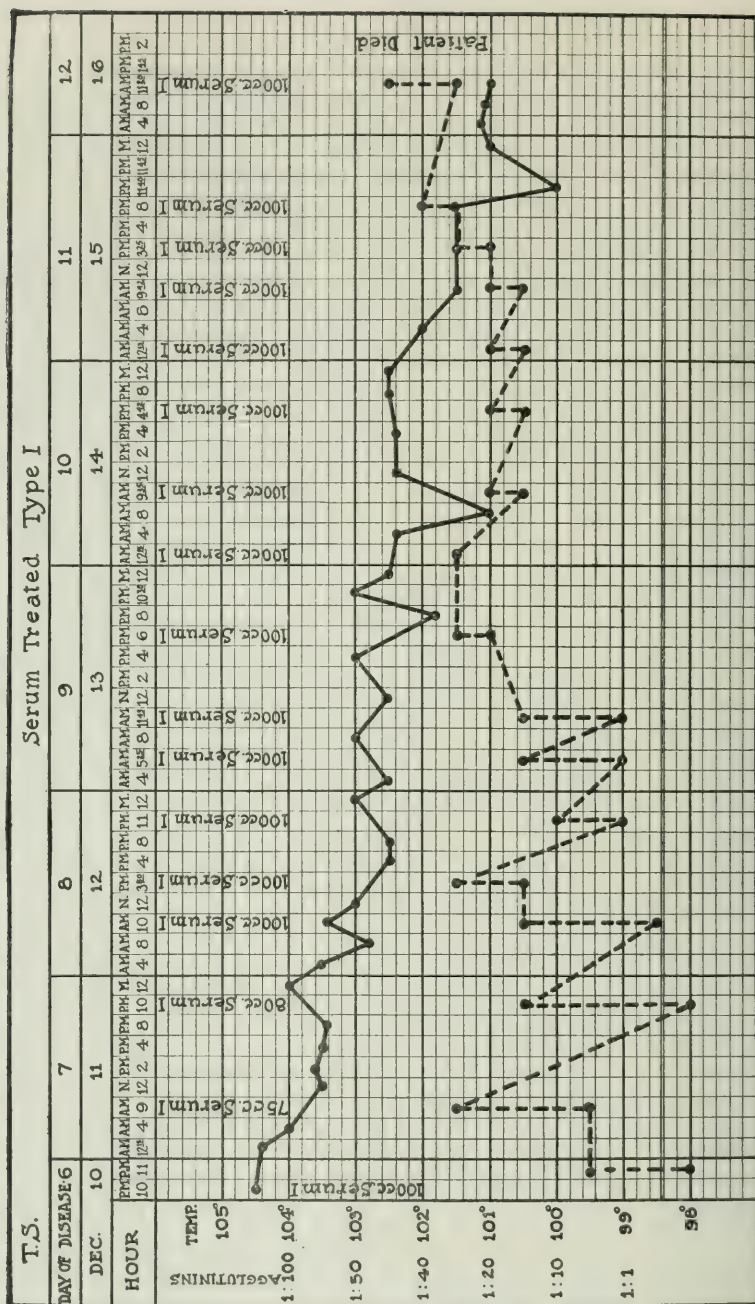
into a mouse, and at 12 midnight, 12 hours after admission, he was given his first treatment. As the curves presented in Text-fig. 5 show, the first dose of serum imparted to the blood a well marked power of agglutination, and each subsequent dose was followed by an increase of this property. With the increase of agglutinins in the blood there occurred an improvement in the patient's condition, the temperature fell, and he finally recovered completely.

The two examples given above illustrate the effects of serum treatment in the cases due to Type I infection, when the serum is given early and in large amounts, and when the infection has not reached too high a grade before the treatments are commenced. Since in twenty-eight out of the thirty cases recovery followed the administration of the serum, we did not have great opportunity to study cases of this type in which the serum was not effective. In all the cases treated with this type of serum agglutinins could be demonstrated in the patient's blood 2 to 3 minutes after the administration of 75 to 100 cc. of serum. The agglutinating power varied somewhat, though in most cases it occurred with a dilution of 1:10 or more. The refinements of the method are not sufficient to justify our calculating in each case the probable dilution and the consequent probable loss in agglutinins in the short interval elapsing before the first tests were made. In general, where the administration of subsequent doses of the serum has not led to a prompt increase of the agglutinins in the blood above the previous level the fall of temperature has been longer delayed and more serum has been required than in the cases in which a regular step-like rise took place. In comparing the temperature and agglutination curves in these cases it has been necessary to keep in mind the fact that the temperature alone does not offer a safe and sure criterion for judging the patient's condition and therefore for the effectiveness of the serum. In view of this fact it has been surprising to see the considerable uniformity with which the temperature and agglutination curves run in opposite directions. With rise of agglutinating power the temperature curve falls.

Of more importance than the immediate rise in agglutinins following the first dose is probably the persistence of the agglutinins in the blood during the subsequent 8 to 10 hours elapsing before the following dose of serum is given. In only five of the cases did a decrease during this period occur. In three of the cases the loss occurred only following the first dose. Following the subsequent doses the concentration reached a high level and persisted. The data in these three cases is not sufficient to enable us to state categorically that this loss indicated a greater severity of infection, though taken in connection with our other observations this seems probable. Two of these three cases were treated on the 4th day and one on the 6th. They

required two, three, and four doses of serum respectively and all made good recoveries following the serum treatment. In one of the other cases in which the agglutinating power disappeared before the following dose was given, the concentration of immune bodies following the first three doses was such that agglutination did not occur with dilutions greater than 1:5, and following the first two doses this power disappeared completely before the subsequent dose was given. It was only after numerous doses had been given that the concentration of agglutinins reached any considerable level and persisted. Altogether this patient required eleven doses of serum given over 7 days. Treatment was commenced in this patient on the 2nd day, but it was not pushed with great vigor at the start, the first dose being 80 cc., with 18 hours elapsing before the administration of the second dose of 70 cc., and 12 hours again elapsing before the administration of the third dose of 80 cc. This case suggested very strongly the inadvisability of inactive treatment at the start. This patient ultimately recovered and there occurred no extension of the lesion to other lobes, but the temperature remained high for 10 days and he was very ill. Finally, the last case in which the agglutinins disappeared between subsequent doses and in which there occurred difficulty in causing a persistent concentration of immune bodies in the patient's blood by the administration of immune serum was one of the two cases which ended fatally. The curves taken from the record of this case are shown in Text-fig. 6. It is apparent from the curves that it was not until treatment had been continued for 3 days, and nine doses had been given, that a persistent concentration of immune bodies at a high level was attained. Even following this there was a constant tendency for the concentration of immune bodies in the serum to fall, rather than to rise.

It should be noted that the treatment in this patient was commenced only on the 6th day, and 11 and 13 hours elapsed between the first and second, and the second and third doses, respectively. He was desperately ill on admission; temperature 104.5°F., pulse 136, and the blood culture showed an extremely high grade of infection, over 300 colonies per cc. In spite of this he lived until the 12th day. It seems that in this case the serum prolonged life. The infection and intoxication, however, at the start were so great that, although the



TEXT-FIG. 6. Chart showing the curve of the agglutinin titer and the temperature curve of T. S.

infection could be kept down, the intoxication could not be recovered from. In this instance it is likely, judging from the experimental observations, that the presence of large amounts of soluble inhibiting substances in the blood prevented the action of the immune serum. It is probable that in such cases very late in the disease these substances may be so large in amount that no practical amount of immune substances can neutralize them. If these conceptions are correct, the importance of giving very large doses of immune serum at the beginning of treatment is apparent.

In the other fatal case, persistent high concentration of immune bodies in the patient's blood was obtained without difficulty. Nevertheless, the patient's condition did not improve and repeated doses of serum were administered. Type I pneumococci had been obtained from the sputum, and the blood culture showed 47 colonies per cc. of the same organisms. Treatment was commenced on the 6th day and the patient died on the 10th day. The pathological changes were extensive in both lungs. The autopsy showed a very widespread tuberculous involvement of both upper lobes and the upper portion of the lower lobe on each side. At the base of one lung, however, was a small area of complete consolidation, differing in appearance from the remainder of the tuberculous lung. This proved on study to be a typical acute diffuse pneumonic process and from it Type I pneumococci were cultivated. We have here an instance in which the serum was apparently effective against the specific infection, but death occurred on account of factors associated with the primary and extensive tuberculosis.

A further interesting case in this connection was one due to Type IV infection. Owing to a mistake in the early determination of the type of infection the patient received several doses of Type I serum before the mistake was discovered. In this patient, although he was quite ill, the administration of the serum caused a prompt appearance of agglutinins in the blood and this increased with the subsequent doses, without any material fall.

These studies of agglutination curves in the cases of Type I infection, however, while instructive and suggestive, do not after all give definite proof that the effect of immune serum is limited by the presence of soluble substances in the blood. When they are con-

sidered, however, in the light of the observations on the Type II cases which follow, the evidence becomes much more suggestive.

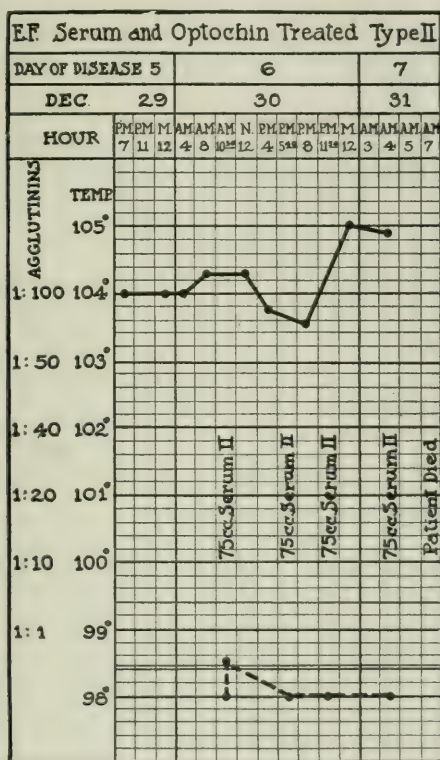
Cases Due to Type II Infection.

The reader should be reminded at the outset that it has been impossible to produce a serum against Type II pneumococci which is as active either *in vitro* or *in vivo* as is the serum against Type I pneumococci. Whereas the horse serum against Type I infection is of such a strength that 0.2 cc. will regularly protect a mouse against 0.1 cc. of virulent culture, it has been impossible to produce an immune Type II serum of any greater activity than that 0.2 cc. will protect a mouse against 0.01 cc. of culture. Moreover, the active Type I sera usually cause agglutination of homologous organisms in dilutions of 1:400 or over; the Type II sera usually cause agglutination in dilutions no greater than 1:200. It should also be noted that the capsule formation of Type II pneumococci is more highly developed than is that of Type I pneumococci. Dochez and Avery⁹ have pointed out that production of precipitable substances in the blood and urine of infected animals apparently bears some relationship to this property of capsule development, the Type III organisms, which possess large capsules, forming most of this substance, the Type II organisms, which have smaller capsules, producing less, and the Type I organisms, which have small capsules, producing still less. While it is not certain that the substances in the infected animals which give rise to fixation of antibodies are identical with those concerned in the precipitation phenomenon, it seems likely that this is the case.

Studies of the agglutinin content of the blood were made in nine cases of Type II infection which received Type II serum. Of these patients, four recovered and five died. Of the patients who recovered, in two treatment was commenced on the 3rd day, in one on the 4th day, and in one on the 5th day. Of the fatal cases, treatment was commenced in one on the 3rd day, in one on the 5th day, and in the others on the 6th day. In all the four cases which recovered, a satisfactory and persistent concentration of agglutinins in the blood appeared. In one of the fatal cases, practically no agglutinins ap-

⁹ Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477. •

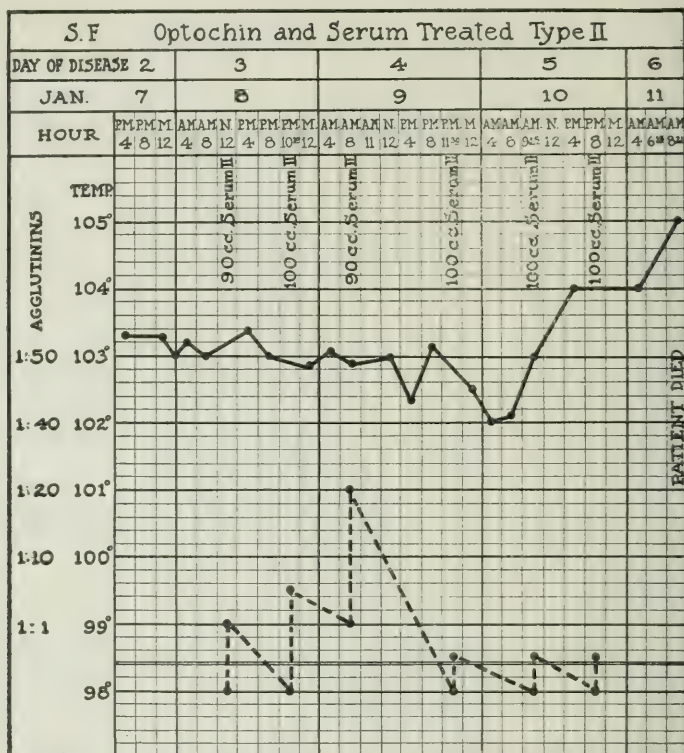
peared in the blood in spite of four doses of serum; in one a satisfactory concentration appeared only after three doses, and then disappeared; in another a satisfactory concentration was obtained only on the 9th day, after five doses of serum; in another, while a satisfactory concentration of agglutinins was obtained on the day



TEXT-FIG. 7. Chart showing the curve of the agglutinin titer and the temperature curve of E. F.

treatment was commenced, the 6th day, the patient was at that time suffering from meningitis, from which he died 2 days later. In the remaining case only one dose of serum was given, 2 hours before death; no agglutinating power appeared in the blood. Text-figs. 7 and 8 show graphically the results of observations made in two of the fatal cases.

Case 1.—E. F., porter; age 46 years. This patient was admitted on the 5th day of the disease with an extensive lung involvement and very severe septicemia, the blood cultures showing 1,000 colonies per cc. His condition was very serious; temperature 104°F., pulse 104, respirations 64, and, in spite of four doses of serum within 24 hours, the patient died. It will be noted that immediately following the administration of 75 cc. of serum, the undiluted blood showed power



TEXT-FIG. 8. Chart showing the curve of the agglutinin titer and the temperature curve of S. F.

of agglutination, but this property had disappeared within 7 hours, and following the subsequent doses the blood showed no agglutinating power whatever. It is evident, therefore, that in this severely infected case the immune bodies in the serum disappeared from the blood as fast as they were administered.

Case 2.—S. F., clerk; age 31 years. This patient, though admitted early in the disease, was extremely ill; temperature 103.3°F., pulse 160, respirations 56. The blood culture showed 400 colonies per cc. and the following morning, before the

first dose of serum was given, the culture showed 1,600 colonies per cc. Following the first two doses of serum there occurred an immediate appearance of agglutinins in the blood in low dilutions, which, however, disappeared or became minimum in amount before the succeeding doses. In spite of the extreme grade of blood infection, the number of organisms present in the blood diminished following these two doses, the cultures on the morning of the 4th day showing only 20 colonies per cc. There was a satisfactory increase in agglutinins following the third dose, but it will be noted that 15 hours were allowed to elapse between this dose and the succeeding one, and during this time the agglutinins had entirely disappeared and the subsequent doses produced little or no effect on the agglutinin content (Text-fig. 8). The patient died on the 6th day.

Five patients suffering from Type II infection who received no serum were also studied to observe the appearance of agglutinins in the blood. All these recovered. In four of these instances at the end of the disease there developed well marked power of agglutination; in one of them agglutination in the serum obtained on the 10th day occurred in a dilution of 1:100. In the fifth no agglutinins appeared in the blood, though this was not studied later than the 12th day. This patient received optochin, as did, however, several of the cases in which agglutinins developed.

DISCUSSION.

Neufeld and Haendel,¹⁰ Dochez,¹¹ and others have shown that specific immune substances usually appear in the blood during recovery from lobar pneumonia. This is shown by an increase in protective power of the blood for mice against homologous infection. Clough¹² has made similar observations and he and others have also noted that in certain instances the protective power is accompanied by the power of inducing *in vitro* phagocytosis of virulent homologous pneumococci which are not phagocytatable in normal serum. It would seem, therefore, that bacteriotropins represent one form of immune body playing a part in this protective phenomenon. Bull¹³ has brought forward experimental evidence which indicates strongly that the phenomenon of agglutination is of great importance in the

¹⁰ Neufeld, F., and Haendel, *Arb. k. Gsndhtsamte.*, 1910, xxxiv, 166.

¹¹ Dochez, A. R., *J. Exp. Med.*, 1912, xvi, 665.

¹² Clough, P. W., *Bull. Johns Hopkins Hosp.*, 1913, xiv, 295.

¹³ Bull, C. G., *J. Exp. Med.*, 1915, xxii, 457.

action of immune serum in pneumococcus infection. It is possible that several different antibodies or phenomena take part in the mechanism of pneumococcus humoral immunity. The observations I have mentioned, as well as unpublished observations made in this laboratory, indicate strongly that natural recovery in pneumonia is associated with the development of humoral immunity and probably occurs because of this development. In the individual case, however, the factors which determine recovery or death cannot be stated so simply. In mild cases probably a very slight grade of humoral immunity may be sufficient to prevent progress of the disease, a grade of immunity which can be detected with difficulty by our present means. In other instances the reaction required on the part of the body may be very great and the immunity phenomena exhibited by the serum when tested outside the body may be very vigorous and marked.

The phenomenon of agglutination offers one ready means for testing the degree of humoral immunity. It is, however, not the only one and it is unsafe to judge of the immunological effectiveness of a serum solely by its agglutinating strength. The protective power and agglutinating power of immune horse serum, however, tend to run parallel. Consequently, the study of agglutinating power of the blood of patients, such as has been made in the present instance, must be of considerable value in indicating the presence or absence of humoral immunity. If recovery in pneumonia is due to the development of humoral immunity, the study of its appearance during recovery and especially of its appearance following treatment with immune serum, should be of significance. In commencing the study it was thought that the method might be employed to graduate the dosage of immune serum in the treatment of the individual case. If recovery is due to the appearance of immune bodies in the blood, the ideal serum treatment would be such that sufficient serum be administered to produce the required concentration of immune bodies and no more. It soon became apparent, however, that such a method, testing the blood before and after the administration of each dose, involved so much time and labor that it would not be of practical value. It has seemed, however, that the repeated tests of the serum in a series of cases, as has been done here, give us considerable

knowledge of the mode of action of the serum and offer valuable suggestions for the routine dosage and mode of application of the serum. The studies have further indicated strongly that during infection not only must sufficient immune substances be added to bring about a concentration sufficient to sensitize all the bacteria, produce their agglutination, opsonification, etc., but in addition there must be a sufficient amount administered to neutralize any soluble substances present in the serum which have the property of neutralizing and fixing the immune substances. It is realized that the occurrence of these soluble, fixing substances in the blood of infected patients has not been directly demonstrated. The experimental observations in animals previously described, however, make it altogether probable that these substances are present in severe infections. It must be admitted that in most instances where there was failure of immune substances to appear in the blood, or where the immune bodies disappeared very rapidly following their administration, bacteriemia was shown to be present before the first dose of serum was administered. In several cases, however, the blood infection could not be demonstrated after the first dose, and nevertheless, rapid disappearance of the immune bodies occurred following the subsequent doses. In one instance in which the rapid disappearance of immune bodies occurred, the blood cultures taken both before and after the administration of serum were sterile. However, it seems likely that in all cases when fixation of immune bodies occurs, blood infection has at some time been present, though the possibility that the fixing substances may, in certain instances, arise entirely in local foci cannot be excluded.

The nature of the substances bringing about the fixation can at present only be conjectured. The demonstration, however, by Dochez and Avery⁹ of substances giving rise to precipitates in the blood and urine of infected patients makes it probable that the same substances are responsible for the phenomenon of fixation that we have studied. They have apparently shown that these substances may be excreted or formed by the bacteria during their growth, and it is also probable that substances contained in the bacteria and set free during their dissolution may give rise to the same phenomenon.

The observations made in this study have a practical bearing on the

question of the therapeutic administration of immune serum. The amount of serum necessary to be given does not depend merely on the weight of the patient and therefore on the consequent dilution of the serum in the body. It is also not entirely dependent on the degree of infection present. If the patient is treated early before large amounts of the soluble substance are present, a moderate amount of serum may be sufficient, even though the grade of blood infection may be considerable. On the other hand, if the infection has continued for a considerable time, and large amounts of soluble, fixing substance are present in the blood, the amount of serum required may be very large. It is therefore evident that it is important that the patient be treated as early as possible and before large amounts of these fixing substances are formed. Moreover, the importance of treating very actively at the start in order that all these fixing substances may be at once neutralized and the progress of the infection immediately and entirely overcome is apparent. It is therefore our plan at present to treat all patients with Type I infection with large initial doses, and to repeat the treatment every 6 to 8 hours as long as may be necessary. It is possible that the Type II serum is less effective than Type I serum not only because its concentration of immune bodies is less than that of Type I serum, but also because the power of pneumococci of this type to produce fixing substances is more highly developed than is that of pneumococci of Type I.

CONCLUSIONS.

1. In empyema fluids resulting from infection with pneumococci there are present large amounts of soluble substances which have the property of neutralizing pneumococcus antibodies.
2. Similar substances are found in the blood of infected rabbits.
3. When immune serum is injected into infected rabbits the immune substances disappear very quickly, and therefore are prevented from activity in overcoming the infection.
4. When immune serum is administered to patients severely infected with pneumococci, the immune bodies may also disappear very rapidly, and this disappearance is probably associated with the presence of such soluble substances in the blood.

5. The serum only becomes effective when these substances are neutralized.

6. The study of agglutination curves is of value in showing why in certain instances favorable results have not followed the use of immune serum.

7. It is important that in severely infected patients the serum be administered early in the disease and that the initial dosage be large.

THE ELABORATION OF SPECIFIC SOLUBLE SUBSTANCE BY PNEUMOCOCCUS DURING GROWTH.

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From the study of bacterial infections in man and the lower animals evidence has been accumulated that pathogenic organisms do not produce harmful effects from their mere physical presence. The general reaction or toxemia of infection is differentiated from the local process which in many instances is the only tangible expression of bacterial invasion. Substances of a harmful nature seem to pass out from the bacteria and through the circulating medium of the animal to injure cells and organs at a distance from the site of infection. An explanation of the nature and mode of action of these substances has been one of the great problems in the study of infection. Certain bacteria, such as diphtheria, tetanus, and others, when grown in artificial media form a soluble toxin whose action when injected into animals differs in no way from that manifested when the same bacteria grow in living tissue. The majority of pathogenic bacteria are not known to form these soluble toxins during their life processes. The capacity of the latter to intoxicate has been explained by their setting free upon death a toxin which during life is retained within the cell body. Some investigators hold the view that intoxication by these bacteria arises in the body of the infected animal from the splitting of the bacterial protein into toxic degradation products. These explanations of bacterial intoxication are not so satisfactory nor so well substantiated as are the facts concerning infection with bacteria which produce known soluble toxins.

Pneumococcus is a highly pathogenic microorganism which is not known to secrete a soluble toxin, and whose harmful effects are supposed to be due either to the setting free of intracellular toxins or

to the formation on disintegration of toxic split products. In the present paper it is shown that this organism during the early stages of its growth forms a readily soluble substance which diffuses into the culture medium *in vitro*, and in human and animal infections is present in the circulating blood, whence it passes through the kidneys into the urine. We have not as yet been able to demonstrate with certainty that this substance is responsible for the intoxication that accompanies lobar pneumonia.

Formation of a Soluble Substance in Culture Media.

In 1897 Kraus¹ demonstrated for the first time the presence of specific precipitable substances in the germ-free culture filtrates of certain bacterial species. This work was subsequently extended by other observers so that it is now known that a large number of bacteria give rise to these substances in the media in which they grow. The precipitin reaction obtained with these substances is strictly specific and occurs only when an homologous immune serum is used. These culture fluids have been studied after the bacteria have grown in them for 24 hours or more and their presence has been supposed to be due to the passing into solution of the bacterial substance upon disintegration of the cells. The same precipitable substances may be demonstrated in the bacteria-free salt solution or distilled water extracts of organisms grown on solid media.

Neufeld² has shown that solutions of pneumococcus obtained by the addition of small amounts of bile to bouillon cultures produce a specific precipitate in the presence of immune rabbit serum. Wadsworth³ has obtained similar results not only with bile solutions but also with filtered salt solution extracts of pneumococcus. Panichi⁴ demonstrated the presence of a specific precipitable substance in the filtrate of bouillon cultures of pneumococcus.

The fact which we wish to emphasize in this study is that pneumococcus from the time it starts to grow elaborates in the medium of its environment a specific substance of bacterial origin in considerable amounts and that the early presence of this soluble substance is not attributable to the death and subsequent disintegration of the bacterial cell, but represents the extrusion into the medium of bacterial substance during the life processes of the organism.

¹ Kraus, R., *Wien. klin. Woch.*, 1897, x, 736.

² Neufeld, F., *Z. Hyg. u. Infektionskrankh.*, 1902, xl, 54.

³ Wadsworth, A., *J. Med. Research*, 1903-04, x, 228.

⁴ Panichi, L., *Centr. Bakteriolog., 1te Abt., Orig.*, 1907, xliii, 188.

TABLE I.
Rate of Growth of Culture.

No. of hrs.	Colonies per cc.
0	810,000
2	1,170,000
4	26,000,000
6	245,000,000
8	297,000,000
12	382,000,000
24	No growth from 0.0001 cc.
48	" " " 0.1 "

Titration of Precipitin Reaction in Culture Fluid.

Dilution.	After 0 hrs.	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 12 hrs.	After 24 hrs.	After 48 hrs.
1:1	—*	±	+++					
1:5	—	—	++					
1:10	—	—	±	+++	+++	+++	+++	+++
1:15	—	—	±	+++	+++	+++	+++	+++
1:20	—	—	±	+++	+++	+++	+++	+++
1:25	—	—	±	++	++	+++	+++	+++
1:30	—	—	±	+	++	++	+++	++
1:40	—	—	—	+	+	++	++	+
1:50	—	—	—	±	+	+	+	+
1:60	—	—	—	±	±	±	±	±
1:80	—	—	—	±	±	±	±	±
1:100	—	—	—	—	—	±	±	±
1:120	—	—	—	—	—	±	±	±
1:160	—	—	—	—	—	—	—	±
1:200	—	—	—	—	—	—	—	±
1:240	—	—	—	—	—	—	—	±
Control.	—	—	—	—	—	—	—	—

* — indicates no reaction; ±, faint trace; +, visible precipitation; ++, flocculation; +++, heavy flocculation.

In the following experiment a flask of bouillon was incubated with a small amount of an early, rapidly growing culture of pneumococcus. A young culture was chosen in order to avoid the occurrence of bacterial lag, during which some cell death occurs. At varying intervals during the growth of the culture, fractions were withdrawn from the flask, freed from bacteria, and the cell-free fluid was tested for the

presence of precipitable substances. A bacterial count was made of each specimen in order to determine that the culture was growing at a maximum rate and that little or no cell disintegration had occurred at the time when this substance was already present in considerable amounts. The quantity of precipitable substance present in a given specimen was determined by ascertaining the maximum dilution of the cell-free fluid at which precipitation occurred on the addition of homologous antipneumococcus serum.

A protocol in which Type III pneumococcus was studied is given, since this organism forms a large amount of soluble substance, whereas Type II and Type I form lesser amounts in the order named.

The bacterial counts in the experiment given (Table I) show that the cultures grew at a maximum rate for about 12 hours. Chesney⁵ has shown in an elaborate study that during this period the bacteria increase in geometric progression and that the curve of generation time may be plotted as an ascending straight line. From this the deduction may be drawn that during the first 12 hours little or no cell death occurs. Examination of the precipitin reaction with the bacteria-free filtrates of specimens removed from the culture during the first 12 hours of growth reveals the fact that the bacterial substance passes into solution in the culture medium in easily demonstrable amounts during this time. This would seem to indicate that this soluble substance is not the result of bacterial disintegration but represents an actual extrusion of the cell substance into the medium during the life processes of the organism.

Presence of Soluble Substance Derived from Pneumococcus in the Blood and Urine of Infected Rabbits.

The demonstration that pneumococcus during its growth in fluid media gives rise to a soluble substance suggested the likelihood that the same substance might be detected in the body fluids of experimentally infected animals. In order to test this assumption, a rabbit was injected intraperitoneally with 1 cc. of the blood of a rabbit infected with pneumococcus. At varying intervals after infection specimens of blood were collected from the heart, and the serum,

⁵ Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

freed from cells, was passed through a Berkefeld filter in order to remove the organisms that had reached the blood stream. The bacteria-free serum was then tested for the presence of precipitable substances by the addition of homologous immune serum. The urine of these rabbits was also tested to find out whether the soluble bacterial substance passed through the kidneys and could be demonstrated by the precipitin reaction in the animal's urine. In Table II an example is given in which the rabbit had been infected with a Type II pneumococcus.

TABLE II.

Pneumococcus Precipitin Reaction in the Blood Serum of a Rabbit Infected with Pneumococcus Type II.

Time.	Before infection.		2 hrs. after infection.		4 hrs. after infection.		6 hrs. after infection.		8 hrs. after infection.	
	I	II	I	II	I	II	I	II	I	II
Type serum....	I	II	I	II	I	II	I	II	I	II
Result.....	—*	—	—	±	—	+±	—	++	—	++

* — indicates negative; ±, faint trace; ++, marked flocculation.

Tests of rabbit urine cannot be made at regular intervals because of the failure of the animal to void frequently. Specimens at the end of 24 hours, however, showed a marked precipitate when mixed with the serum corresponding in type with the organism with which the animal was infected.

The experiment given in Table II demonstrates the fact that within a short period of time after intraperitoneal injection of a rabbit with pneumococcus there is present in the filtered blood serum a specifically reacting bacterial substance of pneumococcus origin. This substance readily passes from the blood through the kidneys into the urine and can there be demonstrated in considerable concentration.

Presence of Soluble Substance Derived from Pneumococcus in the Blood and Urine of Patients Suffering from Lobar Pneumonia.

The fact that the pneumococcus forms a readily soluble substance during growth in artificial media and in the body fluids of animals

experimentally infected makes it not unreasonable to assume that the same substance is formed by pneumococcus during the course of natural infection in human beings. In order to find out whether or not this is so, the blood sera and urine of a large number of patients suffering from pneumonia due to pneumococcus of Types I, II, and III were studied for the presence of this soluble material. Specimens of serum were obtained at varying intervals during the disease and the urine was examined frequently throughout the course of the infection and during convalescence. If the precipitin reaction was not positive with the whole urine, a method of concentrating the urine was employed. It has been found that the soluble substance to which the pneumococcus gives rise is precipitated by alcohol and after precipitation is again readily soluble in water. In order to concentrate the precipitable substance in urine to 25 cc. or more of the 24 hour specimen a few drops of acetic acid are added and the urine is then boiled down to a volume of 5 cc., filtered through paper to remove any precipitate of albumin that may occur, and the filtrate added to eight to ten volumes of 95 per cent alcohol. The precipitate which forms is collected by centrifugalization and dried to remove the excess of alcohol and the residue extracted with 2 or 3 cc. of salt solution which redissolves the specific substance. Any insoluble material is removed by centrifuging and the clear salt solution extract used in the precipitin test. Results of this study are presented in Table III.

In Table III are presented the studies on the presence of the specifically precipitable substance in the blood serum during life of a number of patients suffering from lobar pneumonia. Almost all the patients studied showed a strongly positive precipitin reaction in the urine and were chosen for the purpose of finding out whether at a time when the substance was being excreted in largest amounts it could also be demonstrated in the circulating blood. In all, 25 cases were examined, of which 10 were due to infection with Type I pneumococcus, 11 with Type II, and 4 with Type III. Of the Type I infections, none gave a positive precipitin reaction in the serum, although in all but one the urine was positive at the time the tests were made and in three instances the reaction in the urine was heavy, indicating the excretion of the soluble substance in considerable

TABLE III.

Pneumococcus Precipitin Reaction in the Blood Serum during Lobar Pneumonia.

Case No.	Blood test.		Blood culture.	Urine.	Serum treatment.	Result of disease.	Remarks.
	Day of disease.	Result.					
Pneumococcus Type I infection.							
2,821	6	—	—	++	+	Recovered.	Total, 10 cases; 2 died.
2,816	8, 9, 10, 11, 12	—	+	++	+	Died.	
2,901	5, 7, 9	—	+	++	+	"	
2,968	6, 7	—	+	+	+	Recovered.	
2,936	7, 9	—	—	±	+	"	
2,815	4	—	—	+	+	"	
2,824	3	—	—	±	+	"	
2,883	5, 6, 7	—	—	—	+	"	
2,858	3, 4, 5	—	—	+	+	"	
2,891	3	—	—	+	+	"	
Pneumococcus Type II infection.							
2,885	3, 4, 6	—	—	+	+	Recovered.	Blood culture positive on 6th day.
2,845	6	++	+	++	+	Died.	
2,868	4	—	—	—	—	Recovered.	
	7			+			
2,829	1	—	—	—	—	"	
2,879	2	—	+	—	+	"	
	7			+			
2,892	5, 6	+	+	++	—	Died.	
2,834	6, 7, 9, 10, 11	—	+	++	+	"	
2,922	4, 5, 6	—	—3	+	+	Recovered.	
			+7				
3,006	2, 3	—	—	++	—	Died.	
3,031	3, 5, 7	+	+	++	+	"	
2,869	3, 4, 5	+	±	+	+	"	
Pneumococcus Type III infection.							
2,898	5, 6, 7	++	+	++	—	Died.	Total, 4 cases.
2,947	6, 10, 11	±	—	+	—	Recovered.	
2,797		+	—	+	—	Died.	
2,783	7	+	+	+	—	"	

quantity. The failure to demonstrate the substance in blood in Type I pneumonias may be partly attributable to the fact that all these cases were treated with Type I antipneumococcus serum, which is known to cause the disappearance of the substance from the urine in many cases during treatment. It has also been shown by *in vitro* experiments that *Pneumococcus* Type I forms less of the soluble substance than organisms of Types II and III. The sera of 11 cases of Type II pneumonia were studied. The urine reaction was positive in 10 of these, while the precipitin reaction in the blood was positive in 4 instances. Of these 11 cases 7 were treated with Type II serum. Among the 7 serum treated cases, 3 showed a positive precipitin reaction in the blood. Of the 4 cases not treated with serum, only 1 gave a positive blood test. A positive blood culture was obtained in 7 of the 11 cases studied. Of the 4 cases with a positive precipitin test in the serum, all showed a positive blood culture, while of the 7 cases with a negative precipitin test in the serum, 3 had a positive blood culture. All 4 patients showing a positive precipitin reaction in the blood serum died, whereas of the 7 with a negative serum test, 2 died and 5 recovered.

4 cases of pneumonia due to *Pneumococcus* Type III were studied. All gave a positive precipitin test in the blood serum. Blood cultures were positive in 2 instances and the precipitin reaction in the urine was positive in all. The infection was fatal in 3 of the 4 patients.

In Table IV are presented the results of the examination of the urine for the precipitable pneumococcus substance in 88 cases of pneumonia due to the fixed types of pneumococcus I, II, and III. Of these 88 cases, 35 were Type I, 28 were Type II, 8 were Type II (atypical), and 17 were Type III. Repeated tests of the urine were made during the course of the disease from within 12 hours after onset in one instance to the 58th day in another. Of the 35 cases due to Type I infection, 20 were positive and 15 negative. A positive blood culture occurred in 13 of the 35 cases. Among the 13 cases with positive blood culture, 9 showed a positive urine reaction and 4 gave a negative result. Of the 20 cases with positive urine reaction, 2 died; of the 14 negative cases all recovered. All these instances of Type I infection were serum treated. The administration of serum

TABLE IV.

Pneumococcus Precipitin Reaction in the Urine during Lobar Pneumonia.

Case No.	Urine reaction.	Day of disease when urine was first tested.	Day of disease when urine was first positive.	Duration of positive reaction.	Blood culture.	Serum treatment.	Result of disease.	Remarks.
Pneumococcus Type I infection.								
				days				
2,816	++	6	6	6	+	+	Died.	
2,936	+	6	6	18	-	+	Recovered.	
2,968	++	5	5	14	+	+	"	
2,858	+	3	4	1	-	+	"	
2,996	+	3	3	15	-	+	"	
2,891	+	3	3	5	-	+	"	
2,965	+	6	6	16	-	-	"	
2,952	+	2	5	3	-	+	"	
2,804	+	6	6	26	-	+	"	
2,955	++	6	6	26	+	+	"	
2,925	+	1	1	1	+	+	"	
2,908	+	5	13	5	-	+	"	
2,924	+	5	5	22	-	+	"	
2,913	+	5	5	1	-	+	"	
2,945	+	5	5	29	+	+	"	
2,906	+	2	2	31	+	+	"	Delayed resolution.
2,852	+	4	4	2	+	+	"	
2,949	+	8	11	24	-	+	"	
2,901	+	5	5	4	+	+	Died.	
2,917	±	6	-	-	-	+	Recovered.	
2,814	-	2	-	-	-	+	"	
2,944	-	5	-	-	-	+	"	
2,874	-	3	-	-	+	+	"	
2,815	-	4	-	-	-	+	"	
2,824	-	3	-	-	-	+	"	
2,821	-	6	-	-	-	+	"	
2,984	-	7	-	-	-	+	"	
3,020	-	3	-	-	+	+	"	
3,043	-	3	-	-	-	+	"	
3,011	-	5	-	-	+	+	"	
2,883	-	4	-	-	-	+	"	
2,954	-	4	-	-	-	+	"	
2,880	-	3	-	-	+	+	"	
3,019	+	4	8	42	+	+	"	
3,033	-	2	-	-	-	+	(empyema). Recovered.	

TABLE IV—*Continued.*

Case No.	Urine reaction.	Day of disease when urine was first tested.	Day of disease when urine was first positive.	Duration of positive reaction.	Blood culture.	Serum treatment.	Result of disease.	Remarks.
Pneumococcus Type II infection.								
				days				
2,869	++	2	2	2	+	+	Died.	
2,922	+	3	3	40	+	+	Recovered.	
3,006	+	2	2	8	—	—	Died.	
2,885	+	2	2	2	—	+	Recovered.	
2,845	++	5	5	2	+	+	Died.	
2,868	+	4	7	3	—	—	Recovered.	
2,899	—	1	—	—	—	—	"	
2,879	—	2	7	1	+	+	"	
2,892	++	5	5	2	+	—	Died.	
2,834	++	5	5	5	+	+	"	
2,991	+	1	2	58	—	—	Recovered.	
2,854	—	3	5	1	—	—	"	
3,003	+	7	7	13	—	—	"	
2,827	++	3	3	1	+	—	Died.	
2,746	+	3	6	9	+	—	"	
2,881	+	6	6	2	—	—	Recovered.	
2,786	—	6	—	—	—	—	"	
2,896	—	7	—	—	—	—	"	
2,926	—	4	—	—	—	—	"	
2,897	+	3	4	1	—	+	"	
2,886	+	3	3	6	—	—	"	
2,890	+	3	3	4	+	+	Died.	
2,971	+	7	14	10	—	—	Recovered.	
2,825	++	1	1	5	+	—	Died.	
3,031	+	3	3	5	+	+	"	
3,047	+	3	3	?	—	—	Recovered.	
2,937	—	1	—	—	—	—	"	
2,934	—	4	—	—	—	—	"	
Pneumococcus Type II (atypical) infection.								
2,861a	—	3	—	—	+	—	Recovered.	
2,878x	—	4	—	—	—	—	"	
2,822b	—	5	—	—	—	—	"	
2,831x	+	4	4	3	—	+	"	
2,864b	+	8	8	2	+	—	Died.	
2,960x	—	1	—	—	—	—	"	
2,963b	+	4	7	13	—	—	Recovered.	
2,935b	—	1	—	—	—	—	"	

TABLE IV—*Concluded.*

Case No.	Urine reaction.	Day of disease when urine was first tested.	Day of disease when urine was first positive.	Duration of positive reaction.	Blood culture.	Serum treatment.	Result of disease.	Remarks.
Pneumococcus Type III infection.								
2,838	—	3	—	days	—	—	Recovered.	
2,889	—	7	—	—	—	—	"	
2,797	++	2	2	5	—	—	Died.	
2,898	++	5	5	3	+	—	"	
2,947	++	5	5	30	—	—	Recovered (em-pyema).	
2,812	+	2	5	1	+	—	Died.	
2,783	+	7	7	1	+	—	"	
2,919	+	5	6	4	—	—	"	
2,800	+	3	3	3	+	—	"	
2,849	+	3	3	2	+	—	"	
2,927	+	2	6	4	—	—	Recovered.	
2,973	+	3	10	4	—	—	"	
2,911	+	2	2	13	—	—	"	
2,837	—	1	—	—	—	—	"	
2,972	+	1	6	2	—	—	"	
2,485	—	6	—	—	+	—	Died.	
2,918	—	2	—	—	—	—	Recovered.	

Summary of Urine Reaction.

Type.	Total No. of cases.			Per cent positive.	Positive blood culture.	Positive urine reaction.		Negative urine reaction.		Fatal cases showing urine reaction.	
	Examined.	Positive.	Negative.			With blood culture positive.	With blood culture negative.	With blood culture positive.	With blood culture negative.	Positive.	Negative.
I	35	20	15	57.1	13	9	11	4	11	2	0
II	28	20	8	71.4	11	10	10	1	7	10	0
II (atypical)	8	3	5	37.5	2	1	2	1	4	1	1
III	17	12	5	70.5	6	5	7	1	4	7	1
	88	55	33	62.5	32	25	30	7	26	20	2
IV	10	0	10	0	2	0	0	2	8	0	2
Cases of respiratory disease due to organisms not pneumococcus.	14	0	14	0	2	0	0	2	12	0	2

in Type I pneumonia often results in the temporary disappearance of the substance from the urine. Upon cessation of treatment the soluble substance may reappear in the urine.

Of 28 cases of Type II infection, 20 gave positive precipitin reaction in the urine, and 8 were negative. A positive blood culture occurred in 11 of the 28 cases. Among the 11 cases with positive blood culture, 10 gave a positive urine reaction and 1 a negative result. Of the 20 cases with a positive urine test, 10 died; of 8 negative cases all recovered.

Among 8 cases of infection with atypical Type II pneumococcus, 3 gave a positive precipitin reaction in the urine and 5 a negative reaction. Inasmuch as a normal Antipneumococcus Serum Type II was used in determining the presence of soluble substance in the urine of individuals infected with atypical Type II pneumococcus, a lower percentage of positive urine reactions should be expected in this series, since the precipitin titer of normal Type II serum is low for these atypical organisms.

17 cases of pneumonia due to infection with *Pneumococcus* Type III were studied. *Pneumococcus* precipitinogen was demonstrated in the urine of 12 of these instances, and was absent in 5. A positive blood culture was obtained in 6 of the 17 cases. Of the 6 cases having pneumococcus septicemia, 5 showed a positive precipitin reaction in the urine. 7 of the 12 cases giving a positive urine test died, while 4 of the 5 negative cases recovered.

A summary of 88 cases of pneumonia due to the fixed types of pneumococcus shows that the soluble substance of pneumococcus origin was demonstrable in the urine of 55 (62.5 per cent) of these patients at some stage of the disease and in 39 instances was positive on the first examination. Among the 55 cases with a positive precipitin reaction in the urine, 20 had a fatal outcome, giving a mortality of 36.4 per cent, and of the 33 cases with a negative urine test, 2 died; a mortality of 6 per cent. In addition to the 88 individuals suffering from pneumonia due to the fixed Types I, II, and III, 10 cases of Type IV pneumonia and 14 cases of respiratory disease due to other organisms were studied for the presence of a precipitin reaction in the urine. Each urine was tested with standard Antipneumococcus Sera Types I, II, and III. In no instance was a posi-

tive reaction obtained at any stage of the disease, a fact which establishes beyond doubt the specificity of the reaction.

In addition to the presence of the soluble substance of pneumococcus origin in the blood and urine, it has also been found in other body fluids. In certain cases it can be readily demonstrated in pleural fluids and pericardial exudates and in the spinal fluid of pneumococcus meningitis.

Certain facts have been ascertained concerning the chemical characteristics of this substance. The specific substance is not destroyed by boiling. It is readily soluble in water and is precipitable in acetone, alcohol, and ether, after which it may be easily redissolved in water. It is precipitated by colloidal iron, and does not dialyze through parchment. The immunological reactions of the substance are not affected by proteolytic digestion with trypsin and it is not split by urease. The determination of total nitrogen and nitrogen partition on the active substance obtained by repeated precipitation with acetone and alcohol shows the substance to be of protein nature or to be associated with protein.

One of the chief points of interest in the discovery of the soluble substance of pneumococcus is whether this substance is in any way responsible for the intoxication which attends pneumococcus infection. Studies to ascertain the answer to this question are being actively carried on at the present time but have not as yet progressed to the point at which a definite answer can be given. It may be said, however, that its toxicity is in no way comparable to that of diphtheria toxin. On the other hand, it possesses a degree of toxicity which, exhibited throughout the course of an infection, may account for the signs of intoxication in lobar pneumonia.

DISCUSSION.

The preceding experimental data have shown that a specifically reacting substance of pneumococcus origin occurs in the bacteria-free filtrates of young cultures of pneumococcus and also in the blood serum and urine of patients during lobar pneumonia. The occurrence of specifically precipitable substances in the cell-free filtrate of bacterial cultures has been known ever since the early obser-

vation of Kraus. It has been abundantly confirmed by other investigators with a variety of bacteria. In general, the presence of this precipitable substance has been demonstrated in culture fluids so old that an opportunity has occurred for cell death and disintegration and consequent solution of bacterial protein. In this paper it is shown that there is present in solution in the culture fluid in which pneumococcus is grown, a soluble substance in considerable amounts at a time when no cell death or disintegration has occurred. Consequently this substance does not represent dead dissolved bacterial protein, but the elaboration and passage into solution of a substance which is the product of the life activity of the cell. In addition to the evidence already cited in support of this fact, it has been demonstrated that the soluble substance is present in culture fluids in considerable concentration at a time when no hemolysin is present. This pneumococcus hemolysin is an intracellular body which does not appear in culture fluids until destruction of the bacterial cell has taken place; hence if the soluble substance described were purely of intracellular origin the curve of its concentration in culture fluids would be coincident with that of the hemolysin. This, however, is not the case, for the curve of hemolysin does not begin to rise until a time when the curve of the soluble substance has almost attained its maximum elevation.

The formation of a soluble substance by the pneumococcus on growth *in vitro* suggested the probability that an analogous substance would be formed on growth of the organism in the animal body and because of the readiness with which the substance passes into solution one would expect no difficulty in demonstrating it in the body fluids of experimentally infected animals. Examination of the blood and urine of rabbits infected with pneumococcus has shown this substance to be present in considerable quantities following intraperitoneal infection. Ascoli and Valenti⁶ have demonstrated in the organisms of animals infected with anthrax a substance specifically precipitable with antianthrax serum. Bail⁷ has shown the presence of a substance in the exudates of animals infected with anthrax which, when the fluids were freed from bacteria, increased the infectious

⁶ Ascoli, A., and Valenti, E., *Centr. Bakteriolog., 1te Abt., Ref.*, 1911, xlviii, 243.

⁷ Bail, O., *Arch. Hyg.*, 1905, lii, 272; 1905, liii, 302.

power of anthrax bacilli. This substance he has called aggressin and he considers it to be an excretory product of the anthrax bacillus which favors the invasion of animal tissues by this organism. It is possible that the substances described by Ascoli and Valenti, and Bail are similar in the mechanism of their formation to the soluble substance produced by the pneumococcus. Although our study of other bacteria has been rather limited, it has been demonstrated that certain other species, such as meningococcus, *Bacillus typhosus*, and *Bacillus dysenteriae*, also give rise to soluble material during their growth in fluid media.

A study of the serum of patients suffering from lobar pneumonia has shown that this soluble specific substance is also present in the circulating blood during the course of the disease in man. It gives a specific precipitin reaction with antipneumococcus serum corresponding in type to the organism with which the individual is infected. This soluble precipitable substance in human serum is less frequently present in demonstrable quantities than in the serum of experimentally infected animals. However, it has been found both when pneumococci are present in the circulating blood and when by blood culture organisms are absent. Complement fixation, as well as the precipitin reaction, may be used for the demonstration of this substance in serum. Although the soluble substance is relatively infrequently present in demonstrable quantities in the circulating blood, it is not unlikely, from the fact that the substance appears in a much larger percentage of cases in the urine, that it is much more commonly present in the blood than observed, but in quantities that are below the threshold of demonstration.

A study of the urine in 112 cases of lobar pneumonia and closely related respiratory diseases has shown that in 62.5 per cent of pneumonia due to *Pneumococcus* Types I, II, and III, a substance is excreted in the urine which reacts specifically with antipneumococcus serum of the type corresponding to the organism with which the individual is infected. This substance may appear as early as 12 hours after the initial chill, or may appear for the first time at a later stage of the disease, and may continue to be excreted for many days after recovery has occurred. In certain instances in which excretion occurred over a long time, its persistence in the urine could

be explained by delayed resolution, a condition which represents the passage of the acute pneumococcus infection of the lung into one of a more chronic character. In other instances of continued excretion not explainable on these grounds, the substance must have been stored in the tissues and must have passed into the circulating blood to be excreted by the kidneys without loss of its specific character. It is the rule to find the substance in the urine when pneumococcal septicemia exists. The amount of precipitable substance in the urine seems to be a measure of the severity of the infection. This fact may be dependent upon the quantity of the substance being directly proportional to the actual amount of infection or it may be that the amount of this substance formed bears some relationship to the virulence of the particular strain of pneumococcus responsible for the infection. Most of the instances which fail to show the presence of a precipitable substance in the urine recover, whereas the mortality is high among those in which its presence is demonstrable. If large amounts are excreted the outcome is usually fatal, unless this result is prevented by the administration of anti-pneumococcus serum. The specific precipitin test in the urine is therefore of considerable prognostic value. It may also be used in making a rapid diagnosis of the type of organism with which an individual is infected and in our experience a positive test in the urine is quite as reliable as the agglutination of the organism isolated from the sputum. The precipitin test in the urine, however, should not supplant the usual diagnostic technique in the determination of the type of pneumococcus.

11 years ago Fornet⁸ claimed to have demonstrated in the serum and urine of patients suffering from typhoid fever a substance specifically precipitated by antityphoid serum. From what we now know it would seem likely that his observations were correct despite the fact that subsequent investigators failed to confirm them.

Ascoli⁹ has shown that precipitinogen may pass the kidneys and appear in the urine where it exhibits its specific reaction. We have been able to show that if rabbits are inoculated intravenously with

⁸ Fornet, O., *Münch. med. Woch.*, 1906, xxxviii, 1862.

⁹ Ascoli, M., cited in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, ii, 750.

soluble pneumococcus material a specific precipitin reaction can be obtained in the urine within 24 hours and that the specific substance continues to be excreted for a number of days. In such an experiment the material injected contained no formed living pneumococci. Pettit¹⁰ has demonstrated that if rats are injected with diphtheria toxin, this substance can be shown to be excreted in the urine in active form following the inoculation.

SUMMARY.

1. A specifically reacting substance of bacterial origin is present in the cell-free fluids of young cultures of pneumococcus. This substance is present when the organisms are growing at their maximum rate and undergoing little or no cell death, and consequently its presence is not dependent upon cell disintegration but represents the extrusion of bacterial substance by the living organism.

2. The blood and urine of rabbits experimentally infected with pneumococcus contain a similar specific soluble substance during the early hours of the infectious process.

3. Human beings suffering from lobar pneumonia have in their blood and more frequently in their urine a specific soluble substance of pneumococcus origin. The amount of this substance present in the urine varies in different individuals and the presence of a large amount is of unfavorable prognostic import. This specific precipitin reaction in the urine is of diagnostic value.

4. Rabbits injected with soluble pneumococcus material continue to excrete this substance for a considerable period of time.

5. The specifically soluble substance obtained from bacterial cultures and from the urine during infection is not destroyed by boiling, by precipitation with alcohol, acetone, or ether, or by trypsin digestion.

6. Studies are in progress at this time on the degree of toxicity and on the antigenic properties of the substance.

¹⁰ Pettit, A., *Ann. Inst. Pasteur*, 1914, xxviii, 663.

ACIDOSIS AND ACID EXCRETION IN PNEUMONIA.*

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During the course of certain studies of the various factors of acid excretion,¹ 24 hour samples of urine from several normal and pathological subjects which were examined demonstrated that, in the absence of β -hydroxybutyric acid, the hydrogen ion concentration and titratable acidity are due largely to the ratio between acid and basic phosphates. Although in the main this hypothesis was quite well supported in the twenty-three samples analyzed, in one fatal case of pneumonia, over 60 per cent of the titratable acid could not be accounted for by the phosphates. This observation led to a more extended investigation of acidosis and acid excretion in acute lobar pneumonia, the results of which are presented in this paper.

There exists certain evidence which supports the idea that the metabolism during the febrile stage of pneumonia results in the production of considerable amounts of acid substances. Increased ammonia and titratable acid in the urine have been observed. Diminished carbon dioxide in the blood has been reported by several observers, notably by Peabody² who reviews the literature of the subject. Recently Lewis³ has found the blood of patients with pneumonia to have a decreased affinity for oxygen, a characteristic of acidosis. Palmer and Henderson⁴ noticed that during the fastigium of the disease larger amounts of sodium bicarbonate by mouth were necessary to reduce the acidity of the urine than is normally the case. Such facts as these, increased ammonia and acid excretion, low carbon dioxide in the blood, diminished affinity of the blood for oxygen, and retention of large amounts of alkali, indicate an excessive acid production during the febrile stage of the disease.

* A brief report of this work was given before the American Society for Clinical Investigation, Atlantic City, N. J., May 7, 1917.

¹ Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1914, xvii, 305; 1915, **xxi**, 37; *Arch. Int. Med.*, 1915, xvi, 109.

² Peabody, F. W., *J. Exp. Med.*, 1912, xvi, 701.

³ Lewis, T., *Lectures on the Heart*, New York, 1915.

⁴ Palmer and Henderson, *Arch. Int. Med.*, 1913, xii, 153.

Methods.

The several factors determined in the urine were the hydrogen ion concentration by the colorimetric method previously described,⁵ titratable acidity by the method to be described presently, ammonia by Folin and Macallum's method,⁶ and the total phosphates by the uranium acetate method. The combined carbon dioxide in the plasma was determined by the Van Slyke-Stillman-Cullen method.⁷ As the method of titrating the urine has been modified for a special purpose and, so far as known, has not been used before, it will be described in detail. In the earlier part of the work the method as described by Henderson and Palmer¹ was used, but on account of the variation in the hydrogen ion concentration of the urine and because of the especial interest in the fluctuation in the difference between the total titratable acidity and the phosphate acidity of the urine, it seemed desirable to determine the titratable acidity between two fixed reactions, or in other words to estimate the quantity of acid or base involved in a specified change of hydrogen ion concentration at a level which is a function of the acids in question. By doing this it is possible to compare these differences at a fixed hydrogen ion concentration.

The hydrogen ion concentration⁸ of 5.0 was selected as the acid end-point because most acid urines in pneumonia, while not infrequently reaching this degree of acidity, seldom exceed it. The hydrogen ion concentration of 7.4 as the neutral point was for obvious reasons retained as in the titration previously described. Two titrations are necessary and are carried out as follows: 10 cc. of a 0.1 molal solution of monopotassium phosphate, 4 parts, and disodium phosphate, 25 parts

⁵ Henderson and Palmer, *J. Biol. Chem.*, 1912-13, xiii, 393.

⁶ Folin, O., and Macallum, A. B., *J. Biol. Chem.*, 1912, xi, 523.

⁷ Van Slyke, D. D., Stillman, E., and Cullen, G. E., *Proc. Soc. Exp. Biol. and Med.*, 1914-15, xii, 165.

⁸ Throughout this paper the hydrogen ion concentration has been expressed by the negative exponent of the hydrogen ion concentration, that is, Sørensen's pH. The minus sign is omitted for convenience; for example, a hydrogen ion concentration of 5 means a pH of 5 or an H^+ of 10^{-5} . It should be remembered that an actual increase in hydrogen ion concentration is indicated by a decrease in this exponential figure.

(yielding a hydrogen ion concentration of 7.4) are introduced into a 250 cc. flask, diluted to 250 cc. with distilled water, and 0.2⁹ cc. of a 1 per cent aqueous solution of neutral red is added. 10 cc. of urine are similarly diluted, neutral red is added, and 0.1 N sodium hydrate is run in until the color matches that of the phosphate solution. The second part of the titration is accomplished by introducing 10 cc. of a 0.14 molal solution of acetic acid, 23 parts, and sodium acetate, 46 parts (yielding a hydrogen ion concentration of 5.0) into a 250 cc. flask, diluted to 250 cc. with distilled water, and five drops of a 1 per cent aqueous solution of sodium alizarin sulfonate are added. 10 cc. of urine are similarly diluted, sodium alizarin sulfonate is added, and 0.1 N hydrochloric acid run in until the color matches that of the acetic acid-sodium acetate solution. In matching the color of the alizarin solutions, especially when the urine is highly colored, it has been found desirable to place a flask containing 10 cc. of urine diluted as for titration but without indicator, behind the flask containing the standard acetic acid-sodium acetate solution with indicator. Also a flask with distilled water is similarly placed back of the urinary sample which is being titrated. The titration must be carried out in front of a window with good light. The titratable acidity in 0.1 N cc. between the hydrogen ion concentration of 5.0 and the hydrogen ion concentration of 7.4 is the sum of these two titrations.

With the above data available, it is possible to compare the actual titratable acidity with that which the phosphates alone would give. The calculation of the part of the phosphates is simple. In the equilibrium $10^{\text{H}^+} = K \times \frac{\text{HA}}{\text{BA}}$ approximately, where H^+ is the hydrogen ion concentration, K the ionization constant k for the acid divided by α , the degree of ionization of the salt of the acid, HA the undissociated acid, and BA the salt of the acid, H^+ is determined and K is known, hence $\frac{\text{HA}}{\text{BA}}$ may be easily calculated; and if the total phos-

⁹ It has been found convenient and satisfactory to use a dropping bottle for adding the indicator. Care should always be taken to add the same size and number of drops to standard and urine samples. Three to five drops are sufficient.

¹⁰ Henderson, L. J., *Am. J. Physiol.*, 1908, xxi, 427.

phates are known the amount of HA and BA can be estimated. Reference to Sørensen's measurements shows that this titration is sufficiently accurate within the ranges of hydrogen ion concentration involved in the present investigation. It is appreciated, however, that the presence of electrolytes and variation in the concentration of the urine combine to decrease the accuracy of the titration but not to a degree sufficient to alter the conclusions reached. An example of the calculation follows.

The $\text{pH} = 5.3$ or $\text{H}^+ = 50 \times 10^{-7}$, titratable acidity amounts to 200 cc. 0.1 N acid, total phosphates in terms of phosphorus pentoxide is 1.0 gm. When the acid is monobasic phosphate $K = 2.5 \times 10^{-7}$. We have then, from the above equilibrium, $50 \times 10^{-7} = 2.5 \times 10^{-7} \times \frac{\text{HA}}{\text{BA}}$, whence $\frac{\text{HA}}{\text{BA}} = \frac{20}{1}$; that is,

96 per cent of the phosphate exists as the monobasic phosphate. 1 gm. of phosphorus pentoxide corresponds to 141 cc. of 0.1 N monobasic phosphate. Hence in a mixture of mono- and dibasic phosphates containing 1.0 gm. of phosphorus pentoxide and having a hydrogen ion concentration of 5.3, 135 cc. exist as the acid and 6 cc. in the basic form. As our method of titrating the urine carries

the reaction to a hydrogen ion concentration of 7.4 or when the ratio $\frac{\text{MH}_2\text{PO}_4}{\text{M}_2\text{HPO}_4} = \frac{4}{25}$, 14 per cent of the phosphate is in the acid form, then the per cent of the acid

phosphate which actually takes part in the titration from a hydrogen ion concentration of 5.3 to a hydrogen ion concentration of 7.4 is $96 - 14$ or 82 per cent. Hence 82 per cent of 141 cc. amounts to 115 cc. and the difference between the actual acidity and acidity calculated from the phosphates is $200 - 115$ or 85 cc.

This difference is probably due largely to free organic acids and will be so designated in the discussion following. In Table I the percentage of acid phosphate for any hydrogen ion concentration between 5.0 and 7.4 has been computed, as well as the amounts of acid phosphate entering into the titration between any hydrogen ion concentration within these limits and 7.4.

TABLE I.

Ratio of Acid, HA, to Basic, BA, Phosphate at a Hydrogen Ion Concentration Varying between 7.4 and 4.9 with the Percentage of Acid Phosphate Taking Part in Titration When Carried to a Hydrogen Ion Concentration of 7.4. $K = 2.5 \times 10^{-7}$. $pH = 7.4$ Which Is the Reaction of Normal Blood.

pH.	$H^+ \times 10^{-7}$.	$\frac{HA}{BA}$	HA.	HA-14.
			<i>per cent</i>	<i>per cent</i>
7.4	0.4	$\frac{4}{25}$	14	0
7.2	0.6	$\frac{6}{25}$	19	5
7.0	1.0	$\frac{10}{25}$	29	15
6.9	1.3	$\frac{13}{25}$	34	20
6.8	1.6	$\frac{16}{25}$	39	25
6.7	2.0	$\frac{20}{25}$	45	31
6.6	2.5	$\frac{25}{25}$	50	36
6.5	3.2	$\frac{32}{25}$	56	42
6.4	4.0	$\frac{40}{25}$	62	48
6.3	5.0	$\frac{50}{25}$	67	53
6.2	6.3	$\frac{63}{25}$	72	58
6.1	8.0	$\frac{80}{25}$	76	62
6.0	10.0	$\frac{100}{25}$	80	66
5.9	13.0	$\frac{130}{25}$	84	70
5.8	16.0	$\frac{160}{25}$	87	73
5.7	20.0	$\frac{200}{25}$	89	75
5.6	25.0	$\frac{250}{25}$	91	77

TABLE I—*Concluded*

pH.	$H^+ \times 10^{-7}$.	$\frac{HA}{BA}$	HA.	HA-14.
			<i>per cent</i>	<i>per cent</i>
5.5	32.0	$\frac{320}{25}$	93	79
5.4	40.0	$\frac{400}{25}$	94	80
5.3	50.0	$\frac{500}{25}$	95	81
5.2	63.0	$\frac{630}{25}$	96	82
5.1	80.0	$\frac{800}{25}$	97	83
5.0	100.0	$\frac{1000}{25}$	98	84
4.9	120.0	$\frac{1200}{25}$	98	84

OBSERVATIONS.

For a preliminary survey 24 hour samples of fresh urine from six normal individuals and seventeen cases representing a wide variety of pathological conditions selected from the medical wards of the Massachusetts General Hospital were examined, the results of which appear in Table II.

Case 15 stands out very prominently with 323 cc. of 0.1 N free organic acid, while of the remaining cases 10 showed less than 50 cc., 7 less than 100 cc., 4 less than 150 cc., and 1 normal individual showed as much as 187 cc. It is possible that the high value in Case 6 may be explained partly by the high hydrogen ion concentration and partly by the size of the individual, or, indeed, by a possible abnormality in metabolism. Cases 16 and 23 had high temperatures but showed only small amounts of free organic acid when compared with the total acidity. The ammonia excretion in Case 15 is the highest in the series but is not excessive.

TABLE II.
Normal and Pathological Individuals.

No.	Volume of urine.	Weight.	pH.	Acid 0.1 N.	Ammonia 0.1 N.	Acid + ammonia 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Free organic acid at the determined pH,* 0.1 N.	Remarks.
	cc.	kg.		cc.	cc.	cc.	gm.	cc.	cc.	
1	1,420	94	6.0	336	450	786	2.45	228	108	Normal.
2	1,560	64	6.1	272	375	647	2.31	202	70	"
3	2,000	79	6.3	320	590	910	3.95	295	25	"
4	2,100	89	6.7	143	458	601	2.33	102	41	"
5	1,100	73	5.7	386	418	804	3.05	323	63	"
6	1,000	91	5.4	415	350	765	2.02	228	187	"
7	2,000	61	5.9	186	280	466	1.34	132	54	Chronic alcoholism.
8	740	80	6.1	126	420	546	1.55	135	-9	Arteriosclerosis.
9	1,700	68	5.7	180	293	473	1.40	148	32	Pernicious anemia.
10	2,650	69	7.0	64	382	446	2.16	46	18	Cardiorenal disease.
11	750	78	5.3	125	252	377	0.63	72	53	" "
12	1,290	44	6.0	184	160	344	1.45	135	49	Pleurisy with effusion.
13	2,440	50	6.3	176	346	522	2.02	151	25	" " "
14	2,500	51	6.0	182	390	572	1.58	147	35	Unexplained edema of lower legs.
15	2,360	70	5.3	580	750	1,330	2.25	257	323	Acute lobar pneumonia.
16	580	62	5.3	242	550	792	0.90	103	139	" endocarditis. Temperature 103°F.
17	1,540	68	5.5	510	595	1,105	3.38	377	133	Diffuse sclerosis of central nervous system.
18	1,120	66	6.3	210	392	602	1.95	146	64	Cholelithiasis.
19	1,350	74	6.2	155	141	296	1.18	97	58	Convalescent; typhoid fever.
20	560	63	5.6	145	170	315	0.88	96	49	Cholelithiasis.
21	2,500	50	6.7	100	380	480	1.20	52	48	Chronic constipation.
22	900	57	5.7	206	355	561	1.10	116	90	Diabetes (ferric chloride reaction negative).
23	1,775	60	5.8	445	683	1,128	3.24	334	111	Typhoid fever. Temperature 104.0°F.

* All samples gave no color with ferric chloride.

Normal Individuals.—Four normal subjects of widely varying weights were examined. Very little difference between the individuals was found, hence only one of the protocols is given (Table III).

TABLE III.

Factors Determined on a Normal Individual, Weight 70 Kilos, for 9 Consecutive Days.

Volume of urine.	pH.	Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 5.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.
cc.		cc.	cc.	gm.	cc.	cc.	cc.	cc.	cc.
1,080	5.5	318	378	1.63	182	190	136	188	346
1,270	5.6	324	414	1.81	196	212	128	202	432
1,000	5.6	320	400	1.86	202	220	118	180	280
1,090	5.5	175	251	1.70	189	202	- 14	49	344
600	5.5	180	246	1.00	112	118	68	128	132
1,070	6.6	257	460	1.92	96	228	161	232	293
1,930	6.4	250	425	1.52	103	180	147	245	367
1,015	6.0	142	234	0.85	79	100	63	134	238
1,510	5.7	288	378	1.72	180	202	108	176	417

There is considerable variation in the amounts of free organic acid both when calculated from the hydrogen ion concentration at which the urine was passed and when estimated at the hydrogen ion concentration of 5.0. The hydrogen ion concentration of the case selected varies very little although the free organic acid varies considerably. In general, as the hydrogen ion concentration increases in value, that is, as the urine becomes more alkaline, the amount of organic acid present in the free state is less. On the other hand, as the reaction approaches a hydrogen ion concentration of 5.0 the organic acid fraction increases very rapidly.

Individuals with Acute Lobar Pneumonia.—In all, thirty cases of acute lobar pneumonia, involving the analysis of 325 24 hour samples of urine, were studied. It is not necessary to report in detail all of the protocols of the series. Certain cases have been selected as representative of the various conditions found in the group (Tables IV, V, VI, VII, and VIII). In general it may be said that the more severe the intoxication the greater the amounts of free organic acid at the hydrogen ion concentration of 5.0 which are present. The type of organism¹¹ was determined in twenty-three of the cases, showing 4

¹¹ Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

with Type I, 11 with Type II, 4 with Type III, and 4 with Type IV.

One of the four with Type I infection showed an increase in the free organic acid but in small amount only. In six of the Type II group there was a very marked increase, in three a moderate increase of the free organic acid, while two were without any significant change. There was no increase in the acid excretion in any of the Type III cases. This finding is not what might be expected *a priori* because this type of infection has proved to have the highest mortality of all, 50 per cent of the cases being fatal. One of the group studied died, the protocol of whom is given in Table V. Two of the Type IV cases showed some increase in the free organic acid excretion; the others did not.

The case reported in Table IV was chosen as an example of those individuals who showed no particular signs of either acidosis or increase in acid excretion from the laboratory or clinical standpoint. The total amount of acid excreted is not excessive; although the ammonia excretion on the 6th and 7th days of the disease just before the crisis is greater than on the days following, it is not large. At no time is the combined carbon dioxide of the plasma below normal. The lower limit of normal given by Van Slyke, Stillman, and Cullen is 55 volume per cent, which corresponds to an alveolar air of about 38 mm. carbon dioxide tension. The acidity of the urine fell at the time of the crisis, and this occurred in most of the cases having a definite crisis.

The protocol of a fatal case appearing in Table V is given to illustrate the unexpected finding in the Type III infection. Although the intoxication in this infection was very great, there was no marked increase in the free organic acid nor was there any other evidence of acid intoxication. The ammonia and acid excretion, as well as the combined carbon dioxide in the plasma, were well within normal limits.

In Table VI are given the data of a case with Type II infection which is a fair example of what frequently occurs in the more severe infections.

At the time of the crisis the amounts of acid not accounted for by the phosphates fall off and quite regularly during convalescence

TABLE IV.

Hospital No. 2,852; Male; Age 33 Years. Process Confined to the Left Lower Lobe.

Type I Infection. Blood Culture Positive. Treated with Serum.

*A Severe Chill Followed the Fifth Treatment, after Which
the Temperature Fell by Crisis.*

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a-d)	g (b-e)	h	i	Remarks.
	°F.			cc.		cc.	cc.	gm.	cc.	cc.	cc.	cc.	cc.	cc.	
5	104.5 102.5	124.54 108.46		1,060	5.4	460	533	2.50	282	296	178	237	650	58.6	175 cc. of antipneumococcus serum.
6	103.6 100.8	106.48 96.40		1,105	5.5	196	265	0.51	57	60	139	205	910	60.6	300 cc. of antipneumococcus serum. Chill.
7	106.5 98.2	154.46 72.30		1,185	5.7	250	440	1.20	127	142	123	298	975		
8	100.5 99.5	88.40 72.28		985	6.1	145	334	0.74	65	88	80	246	630	61.7	
9	99.5 99.2	76.40 60.24		865	6.6	152	372	1.10	43	130	109	242	430		
10	100.4 98.5	76.36 60.28		1,200	6.4	370	665	3.00	204	356	166	309	605		
11	99.5 98.5	78.28 64.20		900	5.8	310	436	1.80	184	212	126	224	790	62.2	
12	100.4 99.0	80.32 74.28		900	5.6	225	336	1.27	138	150	87	186	378		
13	100.4 99.4	88.28 80.20		625	5.6	73	145	0.83	90	98	-17	47	295	69.1	
14	101.6 100.0	100.40 80.20		1,230	5.6	325	450	1.68	182	198	143	252	410		Beginning of serum disease.
15*	102.6 100.5	100.36 80.20		850	5.5	278	356	1.32	147	156	131	200	283		

* This case was followed for a week longer but as there was nothing of note in the data, they are not given.

TABLE V.

Hospital No. 2,593; Male; Age 37 Years. Process at the Entrance of the Right Lower Lobe Extending 2 Days before Death to the Entire Right Lung.
Type III Infection. Blood Culture Positive. Treated with Optochin. Died on the 9th Day of the Disease.

Day of disease.	Maximum and minimum temperature.		Maximum and minimum pulse.		Maximum and minimum respirations.		Volume of urine.		a	b	c	d	e	f	g	h	i	Remarks.
	°F.						cc.		pH.	Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 5.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.	Combined carbon dioxide in plasma. Volume per cent.
3	104.0	102.5	112	102	38	32	1,425	5.8	184	395	1.57	162	186	22	209	427	54.1	
4	104.0	103.0	116	100	42	36	1,675	5.7	218	400	1.71	180	202	38	198	524	59.8	
5	103.0	102.0	112	100	44	32	2,200	5.6	396	600	3.34	363	396	33	204	880		
6	103.0	102.0	112	104	40	32	2,040	5.2	388	470	3.04	352	360	36	110	885	51.3	
7	102.0	102.0	120	102	48	32	1,510	5.2	348	410	2.60	300	308	48	102	800	50.4	Died 2 days later.

keep within normal limits. Early in the investigation before the free organic acid fraction was compared at the fixed point of pH = 5.0, this remarkable decrease was somewhat misleading due to the fact that the hydrogen ion concentration of the urine changed to the nearly neutral point at the crisis. On the 7th day of the disease the free organic acid excreted in 24 hours amounted to 760 cc., while in the days following the limits were between 220 and 351 cc. There was also a marked increase in the ammonia excretion, amounting to 2,050 cc. on the 7th day, rapidly coming down to normal values

TABLE VI.

*Hospital No. 2,865; Male; Age 23 Years. Process Involving the Entire Left Lung.
Type II Infection. Blood Culture Negative. Treated with Optochin.
Recovery.*

Day of disease.	Maximum and minimum temperature.		Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a		b	c	d	e	f (a-d)	g (b-e)	h	i
	°F.				cc.		Acid 0.1 N.		Acid pH 5.0-7.4, 0.1 N.	Phosphorus pent-oxide.	Phosphorus pent-oxide acid 0.1 N, pH 7.4.	Phosphorus pent-oxide acid 0.1 N, pH 5.0-7.4.				
4	106.0	120	48													
	105.4	110	32		1,740	5.2	588		640	1.47	170	174	418	466	432	62.5
5	104.8	116	48													
	103.4	104	40		2,350	5.0	910		910	4.00	474	474	436	436	595	61.4
6	103.0	100	44													
	101.0	84	32		3,510	5.8	990		1,380	7.00	723	828	267	552	1,580	65.3
7	100.5	102	48													
	99.2	72	30		2,300	6.2	660		1,120	3.04	248	360	412	760	2,050	66.8
8	101.2	76	40													
	100.0	70	28		1,555	6.5	202		507	1.32	78	156	124	351	1,130	60.8
10	101.2	80	30													
	99.8	46	24		925	5.8	390		565	2.42	250	287	140	278	694	63.8
11	101.0	76	32													
	98.0	60	24		650	5.6	387		500	2.36	256	280	131	220	700	
12	99.5	68	24													
	99.0	58	20		900	5.8	400		532	2.63	271	312	129	220	510	
13	99.8	72	24													
	98.3	60	20		925	5.5	500		563	2.75	306	325	194	238	495	
14	99.2	64	22													
	98.6	60	20		1,688	5.4	450		515	2.10	236	248	214	267	580	
15*	99.5	96	22													
	99.0	60	20		1,200	5.6	412		460	2.03	220	240	192	220	327	

* This case was followed a week longer but as the data contained nothing of especial interest they are not given.

after the crisis. A decided fall in urinary acidity occurred after the crisis. Throughout the entire course of the disease, however, the combined carbon dioxide in the plasma remained normal, indicating the ability of the organism to cope with the increased acid production.

The case reported in Table VII excreted the largest amount of free organic acid in 24 hours of any of the cases observed. On the 4th day of the disease 1,165 cc. of the total acidity of 1,800 cc. at a hydrogen ion concentration of 5.0 were unaccounted for by the phosphates. The intoxication was intense. That the mechanism for regulating the acid-base equilibrium was sufficient is proved by the combined carbon dioxide in the plasma. Only 2 hours before death it was 62.5 volume per cent, the lowest at any time during the disease.

TABLE VII.

Hospital No. 2,869; Male; Age 31 Years. Process in the Right Lower Lobe. Type II Infection. Blood Culture Positive. Treated with Serum and Optochin. Died.

Day of disease.	Maximum and minimum temperature.		Maximum and minimum pulse.		Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a-d)	g (b-e)	h	i
	°F.															
3*	103.4	120	48													
	103.0	108	32	4,340	5.5	1,600	1,900	9.65	1,070	1,140	530	760	737	64.1		
4*	103.3	124	48													
	102.4	108	30	3,160	5.6	1,380	1,800	5.35	580	635	800	1,165	1,730	64.1		
5*†	104.0	128	52													
	102.0	114	30	1,900	5.9	475	820	2.88	284	340	191	480	688	63.5		

* 190 cc. of antipneumococcus serum.

† Died on the 6th day of the disease. The combined carbon dioxide in the plasma 2 hours before death was 62.5 volume per cent.

TABLE VIII.

*Hospital No. 2,634; Male; Age 42 Years. Process Confined to the Right Lower Lobe.
Type IV Infection. Blood Culture Negative. Treated with Optochin.
Recovery.*

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a-d)	g (b-e)	h	i
	°F.			cc.		cc.	cc.	gm.	cc.	cc.	cc.	cc.	cc.	
7	104.8 103.4	106 90	44 32	1,975	5.3	623	730	4.10	470	486	153	244	1,040	60.7
8	104.6 103.0	96 82	48 30	1,580	5.4	350	440	2.24	252	266	98	174	1,060	60.7
9	104.0 102.5	84 82	46 32	1,775	5.2	370	497	2.62	304	310	66	187	1,200	52.8
10	103.5 100.0	88 72	40 32	2,560	5.7	294	460	2.62	278	310	16	150	1,800	64.5
11	102.5 99.0	70 56	30 24	1,550	5.7	268	392	1.83	193	216	75	176	1,180	68.3
12	99.2 98.6	72 58	32 24	1,415	5.5	318	410	2.20	246	261	72	149	900	
13	99.2 98.6	76 56	32 24	1,630	5.6	394	542	3.02	328	358	66	184	625	
14	99.6 98.6	84 58	26 18	1,665	5.5	372	478	2.45	273	290	99	188	500	
15	99.4 98.6	80 64	20 18	1,715	5.2	394	437	2.50	290	296	104	141	620	54.1
17	99.0 98.6	70 52	24 18	1,490	5.0	540	540	3.12	370	370	170	170	690	
18	99.6 98.8	80 58	18 18	1,300	5.0	430	430	2.50	296	296	134	134	533	

TABLE VIII—*Concluded.*

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a-d)	g (b-e)	h	i
	°F.			cc.		Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pent-oxide.	Phosphorus pent-oxide acid 0.1 N, pH 7.4.	Phosphorus pent-oxide acid 0.1 N, pH 5.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N	Combined carbon dioxide in plasma. Volume per cent.
20	99.6 98.7	80 60	20 18	1,500	5.0	480	480	2.74	325	325	155	155	540	
21	99.6 98.8	80 56	20 18	1,380	5.0	400	400	2.60	308	308	92	92	458	
23	100.0 98.8	80 64	20 20	1,080	5.0	260	260	1.67	198	198	62	62	290	49
24	100.0 99.2	80 56	24 18	1,415	5.0	396	396	2.28	270	270	126	126	315	
25	100.2 99.2	72 60	18 18	1,140	5.0	348	348	2.32	280	280	68	68	413	
27	99.5 99.2	84 70	18 18	1,135	5.0	330	330	1.83	217	217	113	113	330	

The individual with a Type IV infection reported in Table VIII revealed a condition not found in any of the other cases studied. While there were only small amounts of free organic acid excreted during the fastigium of the disease and in convalescence, the ammonia excretion was very high until after the crisis, when the 24 hour values became normal. There was never any significant lowering of the combined carbon dioxide in the plasma.

DISCUSSION.

The facts brought out by the investigation are that in many, usually the more severe, cases of acute lobar pneumonia there are excreted during the fastigium of the disease considerable quantities of an organic acid which is free at a hydrogen ion concentration of 5.0, and that

there is seldom a severe grade of acidosis as estimated by the amount of combined carbon dioxide in the plasma. The nature and biological importance of this organic acid are not without interest. Certain possibilities are suggested. Because of its prevalence in many conditions where there is abnormal metabolism, β -hydroxybutyric acid was searched for, although all specimens examined had a negative or at most a very faint ferric chloride reaction. The ionization constant of β -hydroxybutyric acid is 2×10^{-5} , therefore one-third of the acid is free in a urine with a hydrogen ion concentration of 5.0. Quantitative estimates revealed insignificant amounts. This is not surprising when one considers, for instance, that in Table VI on the 7th day of the disease the acid unaccounted for amounts to 760 cc. and if it were all due to β -hydroxybutyric acid there would be present a total of 24 gm. of the acid, an amount which is seldom encountered except in the more severe grades of acidosis in diabetes. Hippuric acid with an ionization constant of 2.2×10^{-4} , acetoacetic acid with an ionization constant of 1.5×10^{-4} , and lactic acid with an ionization constant of 1.4×10^{-4} whereby only 5 to 7 per cent of an acid can exist free at a hydrogen ion concentration of 5.0 could hardly be expected to account for any considerable quantities of free organic acid. Acetic acid, the ionization constant of which is 1.9×10^{-5} , exists about one-third free at a hydrogen ion concentration of 5.0, hence it becomes a possibility. While uric acid with an ionization constant of 1.5×10^{-6} may be 87 per cent free at a hydrogen ion concentration of 5.0, the total amount of this substance is easily estimated and has never been found to account for more than a few cubic centimeters of the free acid. Nor can the conjugated sulfuric acid be responsible for any large quantities as shown by several ethereal sulfate determinations. The oxy- and other less well known acids are possibilities, but as their ionization constants are not known nothing definite can be stated about them. In Table VIII the acid is apparently a fairly strong one as shown by the high ammonia without much free acid or high total phosphates.

As it has been shown that normally at a hydrogen ion concentration of 5.0 there exists a certain titratable acidity that cannot be accounted for by the phosphates, it is possible that the increase in this value during pneumonia may be due simply to an increase of a normal

constituent of the urine. On the other hand, the possibility of abnormal oxidation products leads one to suspect that there may exist some pathological substances to account for the phenomenon and this in part has been borne out by our investigation. If this is the case, its part, if any, in the intoxication encountered in the disease is of much interest. Considerable investigation of the nature and significance of the increase in the free organic acid production of the urine during pneumonia has been carried out, and will form the subject of a future communication.

SUMMARY.

There is excreted in the urine of subjects ill with acute lobar pneumonia a large amount of organic acid which is free at a hydrogen ion concentration of 5.0.

Acidosis as determined by the combined carbon dioxide in the plasma is seldom, if ever, severe.

FURTHER STUDIES ON THE EPIDEMIOLOGY OF LOBAR PNEUMONIA.

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Previous studies on the epidemiology of lobar pneumonia have shown that although pneumococcus is present in the mouths of about 50 per cent of normal individuals, it is extremely rare to find pneumococcus of Type I or Type II in the normal mouth except in individuals who have been in intimate association with patients suffering from lobar pneumonia due to organisms of these types. The observations of Dochez and Avery¹ suggest that in many instances lobar pneumonia may be due to contact infection. The source of such infection may be either an individual who has recovered from lobar pneumonia but who still harbors in his mouth the infectious microorganism, or a healthy carrier who has acquired the organism from association with a case of lobar pneumonia.

In the present paper are reported, first, the varieties of pneumococcus isolated from cases of lobar pneumonia admitted to the Hospital of The Rockefeller Institute during the past 5 years, second, the varieties of pneumococcus found in the mouths of normal individuals, third, a study of the types of pneumococcus present in the mouth secretions of members of households where cases of Type I and Type II pneumonia have occurred, fourth, the types of pneumococcus obtained from the dust of rooms in which no case of pneumonia is known to have occurred, fifth, the types of pneumococcus obtained from the dust in homes where pneumonia of Type I or Type II has occurred, and sixth, two epidemics of pneumonia.

¹ Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1915, xxii, 105.

Varieties of Pneumococcus Concerned in the Production of Lobar Pneumonia.

During the past 5 years a study has been carried on of the types of pneumococcus isolated from 454 cases of lobar pneumonia admitted to the wards of the Hospital of The Rockefeller Institute. These organisms have been isolated from sputum, from blood cultures, or directly from the lung. In many instances they have been isolated from more than one of these sources and classified in the biological group already described by Dochez and Gillespie.² The results of this classification are shown in Table I.

TABLE I.
Types of Pneumococcus Causing Lobar Pneumonia in 454 Cases.

Pneumococcus.		Incidence.	
			<i>per cent</i>
Type I.....	151		33.26
“ II.....	133		29.29
“ II a.....	6		1.32
“ II b.....	4		0.88
“ II x.....	9		1.98
“ III.....	59		12.99
“ IV.....	92		20.26

The incidence of Types II a, II b, and II x, subvarieties of Type II, described by Avery,³ has only been determined during the last 2 years.

Varieties of Pneumococcus in the Normal Mouth.

Pneumococci were isolated in 116 instances from the saliva of 297 normal individuals in whom no history of contact with a previous case of lobar pneumonia could be obtained. In five instances two types of pneumococci were isolated from the same individual. Pneumococcus Type I was isolated only once; Pneumococcus Type II was not found among these 297 healthy individuals. Among the 39 per cent showing pneumococcus in the mouth secretions, the dis-

² Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

³ Avery, O. T., *J. Exp. Med.*, 1915, xxii, 804.

TABLE II.

Types of Pneumococcus Isolated from the Saliva of Normal Individuals Not in Direct Contact with Lobar Pneumonia.

Pneumococcus.	Incidence.	
		<i>per cent</i>
Type I.....	1	0.8
" II.....	0	0
" II a.....	1	0.8
" II b.....	7	5.8
" II x.....	14	11.6
" III.....	34	28.1
" IV.....	64	52.9
Pneumococcus present.....	116	
" absent.....	181	
	<u>297</u>	

tribution of types is in striking contrast to that which is found among individuals in direct contact with disease. In Table IV are given the results of the examinations of 184 normal individuals known to be in contact with a case of lobar pneumonia due to pneumococci of Types I and II. Among 107 normal individuals known to be intimately associated with a Type I infection, 15 per cent showed pneumococcus of the same type as that causing disease. Of the remaining 77 individuals, all of whom were in direct contact with a Type II infection, this organism was isolated in 5 instances (6 per cent). In all, pneumococci of the more strictly disease-producing types, I and II, were found in 11 per cent of individuals in direct contact with cases of lobar pneumonia due to these types; while among 297 individuals in whom no history of previous contact could be obtained, pneumococci of Types I and II were found in only 0.8 per cent.

The pneumococcus is found in the mouths of many normal individuals. In some the same type of pneumococcus is present constantly, while in others the types may vary from time to time or entirely disappear. In Table III is presented a study of the types of pneumococcus recovered from the saliva of 942 healthy persons.

From the saliva of 942 normal individuals including those in contact with lobar pneumonia one or more types of pneumococcus were

TABLE III.

*Types of Pneumococcus Isolated from the Sputum of Normal Individuals,
Including Those in Direct Contact with Lobar Pneumonia.*

Pneumococcus.	Incidence.	
		<i>per cent</i>
Type I.....	34	7.0
" II.....	22	4.5
" II a.....	1	0.2
" II b.....	26	5.3
" II x.....	47	9.7
" III.....	85	17.5
" IV.....	271	55.8
Pneumococcus present.....	450	
" absent.....	492	
	942	

isolated in 450 instances, or 47 per cent. In 492 individuals no pneumococci could be recovered at the time of examination. Type I pneumococcus was isolated from 34 individuals. 33 of these persons had recently been in close association with a case of lobar pneumonia due to the same type. In only one instance was there no history of contact. From 22 individuals Type II pneumococcus was obtained. 19 of these persons had recently been intimately associated with a case of lobar pneumonia of Type II. It is significant that only once was pneumococcus of Type II a isolated from a normal mouth. Yet this organism was responsible for six cases of pneumonia, or 1.32 per cent. On the other hand, Pneumococcus Type II b was found 26 times and Pneumococcus Type II x 47 times in normal mouths. In view of the relative frequency of Types II b and II x in normal mouths, these organisms, as those of Types III and IV, must be considered as normal inhabitants of the healthy mouth. It is not infrequent to find two types of pneumococcus at the same time in one mouth. But as yet a Type I and a Type II pneumococcus have never been found simultaneously in the same mouth.

Incidence of the Carrier Condition in Normal Individuals in Contact with Patients Suffering from Lobar Pneumonia.

Dochez and Avery¹ have shown that many individuals who come in contact with active cases of lobar pneumonia due to infection with *Pneumococcus* Type I and Type II carry in their mouth secretions a pneumococcus of the same type as that causing the disease with which they have been associated. In order to determine the frequency with which normal individuals acquire these types of pneumococcus, the following study was carried on.

A nurse was sent to the home as soon as possible after a patient suffering from pneumonia due to Type I or Type II had been admitted to the hospital. Specimens of sputum were collected from all members of the household; *i.e.*, all persons who had come in contact with the patient. In Table IV are presented the results of this study. This table includes all the cases which were studied from this point of view. It shows the incidence and type of pneumococcus found among the members of 52 households. From 28 of these homes came patients with lobar pneumonia due to Type I infection. Among the 107 members of these families 16, or 15 per cent, showed Type I pneumococcus. One or more positive contacts were found in 10 (36 per cent) of the homes. Type II pneumococcus was isolated from only 1 individual. 77 individuals from 24 homes in which a case of Type II pneumonia had occurred were studied. From 5, or 6 per cent, a Type II pneumococcus was isolated. In 5, or 21 per cent, of the 24 homes a positive contact was found. A Type I pneumococcus was not found among these individuals. Altogether, from a total of 52 cases of lobar pneumonia due to Type I and Type II, 15, or 28 per cent, gave rise to the carrier condition in at least one of their immediate associates. The total number of households examined was 52, and in 15, or 28 per cent, there were one or more persons who showed pneumococcus of Type I or Type II in their sputum. Of the 184 persons who composed these households 21, or 11 per cent, showed either a Type I or a Type II pneumococcus.

The average carrying period for Type I was 25 days, and for Type II 43 days. The carrying period was roughly estimated by taking the middle date between the last positive sputum and the first negative one. If the contact was still positive at the last examination, a plus sign is added.

TABLE IV.

Incidence of the Carrier Condition in Healthy Individuals in Contact with Lobar Pneumonia.

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				<i>days</i>
2,746	I	Father. Mother. Sister T. " A.	IV No pneumococcus. IV IV	
2,751	II	Wife. Sister. Brother. Brother-in-law.	II x and III No pneumococcus. IV II b and IV	
2,786	II	Husband. Son. Daughter. Friend.	II x IV IV II	48
2,804	I	" Mr. O. " Mrs. O. " G. " H.	IV IV II x IV	
2,814	I	Wife. Brother.	IV II b	
2,815	I	Husband. Daughter R. " H. Friend.	No pneumococcus. I No pneumococcus. II x	6
2,816	I	Wife. Son.	No pneumococcus. I	31
2,821	I	Sister-in-law. Brother. Friend.	No pneumococcus. II II b	Undetermined.
2,824	I	Sister.	No pneumococcus.	
2,825	II	Wife. Son-in-law. Daughter.	II No pneumococcus. II x	48
2,827	II	Wife. Friend.	No pneumococcus. II b and III	
2,834	II	Sister. Niece T. " M. Nephew H. Friend C. " D.	IV IV IV II b and IV II b " III II b " IV	

TABLE IV—*Continued.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				<i>days</i>
2,852	I	Mother. Sister M. " A. Brother L. " I.	No pneumococcus. IV No pneumococcus. " " II x	
2,853	II	Mother. Father. Sister E. " A. Brother.	No pneumococcus. IV No pneumococcus. " " " "	
2,854	II	Wife. Daughter H. " M. " I.	" " " " " " III and IV II	
2,858	I	Friend. Wife. Daughter. Son R. " I.	No pneumococcus. IV II x and IV IV	Undetermined
2,868	II	Mother. Brother E. " A. " W.	II b No pneumococcus. " " " "	
2,869	II	Wife. Daughter A. " I. Brother.	IV No pneumococcus. " " " "	
2,874	I	Friend F. " J. " H.	II x III II x	
2,879	II	Wife.	No pneumococcus.	
2,880	I	Mother. Father. Brother W. " R.	IV No pneumococcus. IV III	
2,881	II	Sister. " Brother. Brother-in-law. Niece.	I and IV IV No pneumococcus. II x and III IV	41

TABLE IV—*Continued.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				<i>days</i>
2,883	I	Son. Daughter. Friend S. " Y.	No pneumococcus. " " " " II x	
2,885	II	Maid J. " A. " M.	IV No pneumococcus. " "	
2,886	II	Sister C. " E. Niece. Brother-in-law.	" " II x No pneumococcus. IV	
2,890	II	Wife.	No pneumococcus.	
2,891	I	Sister. Niece E. " M. Brother-in-law.	" " " " " " " "	
2,892	II	Wife. Daughter X. " M. Son. Boarder.	" " II x III II x No pneumococcus.	
2,896	II	Mother. Sister. Brother.	IV IV II x	
2,901	I	Wife. Friend K.	I II x	80
2,906	I	Husband. Sister. Son.	No pneumococcus. " " " "	
2,908	I	Wife. Brother. Sister L. " A.	" " IV No pneumococcus. I	37
2,913	I	Mother. Brother.	No pneumococcus. IV	
2,917	I	Mother. Father. Brother C. " B. Sister S. " N.	IV No pneumococcus. III No pneumococcus. IV No pneumococcus.	

TABLE IV—*Continued.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				<i>days</i>
2,922	II	Wife.	II	70+
		Daughter I.	No pneumococcus.	
2,924	I	Wife.	" "	
		Daughter K.	" "	
		" L.	I	25
		" A.	No pneumococcus.	
		Son.	IV	
2,925	I	Wife.	III	
		Daughter S.	No pneumococcus.	
		" A.	" "	
		" R.	III	
		Son J.	I	25
		" M.	III	
2,926	II	Friend M.	III	
		" F.	No pneumococcus.	
		" L.	II	7
2,934	II	" R.	No pneumococcus.	
		" N.	IV	
		" H.	II x	
		" C.	No pneumococcus.	
		" K.	" "	
2,944	I	Mother.	I	32
		Father.	II b	
		Husband.	I	28
		Friend M.	No pneumococcus.	
		" McC.	IV	
		Daughter M.	No pneumococcus.	
		" H.	I and III	16
		Son.	No pneumococcus.	
		Sister.	I and II x	17
2,945	I	Mother.	I	7
		Father.	I	35+
		Brother I.	No pneumococcus.	
		" M.	" "	
		" C.	III and IV	
		Sister E.	I	9
		" Y.	No pneumococcus.	
		" S.	" "	
2,946	II	Wife.	" "	
		Son.	IV	

TABLE IV—*Concluded.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				<i>days</i>
2,952	I	Wife.	IV	
		Daughter.	No pneumococcus.	
2,954	I	Brother E.	" "	
		" J.	" "	
		Sister R.	" "	
		Cousin.	" "	
2,955	I	Wife.	I	21
		Son.	II x	
		Daughter.	I	5
2,968	I	Sister-in-law.	No pneumococcus.	
		Brother.	" "	
		Nephew J.	II b	
		" O.	IV	
2,971	II	Maid.	No pneumococcus.	
		Father.	" "	
2,976	II	Mother.	" "	
2,984	I	Brother.	IV	
		Nurse.	IV	
2,991	II	Sister.	IV	
2,996	I	Wife.	II x	
		Son.	No pneumococcus.	
M.M.	II	Wife.	IV	
		Mother-in-law.	IV	
		Nurse.	II x	

Summary.

Type.	No. of pneumonia households examined.	Households in which carriers were found.		Total contacts examined.	Positive contacts.	
			<i>per cent</i>			<i>per cent</i>
I.....	28	10	36	107	16	15
II.....	24	5	21	77	5	6
Total.....	52	15	28	184	21	11

Types of Pneumococci Obtained from Dust of Households in Which No Case of Pneumonia Had Occurred.

The presence of pneumococcus in dust has been a known fact for some time. Netter⁴ in 1897 was the first to recover a definite pneumococcus from dust. Previous workers, notably Emmerich,⁵ demonstrated the presence of Friedländer's bacillus in the dust of a room where there were many pneumonia patients. But at this early date the pneumococcus and Friedländer's bacillus were not clearly differentiated. The occurrence of the pneumococcus in dust has been lost sight of and very little significance has ever been attached to it. Its presence in dust has not been correlated with the occurrence of cases of pneumonia beyond a few casual references to the finding of pneumococcus in the dust of wards where there were many pneumonia patients.

In order to determine whether pneumococcus could be recovered with any regularity from dust the following study was carried out. The specimens of dust were collected as follows: A piece of paper was wrapped about a scrubbing brush which in turn was covered by a piece of cloth and then autoclaved. The dust was swept up with the sterile brush on to the paper which was then folded. This dust was mixed with sterile broth and 1 or 1.5 cc. of the mixture were immediately injected into the peritoneal cavity of a white mouse. Cultures were made from the heart's blood of the mouse.

In Table V are given the types of pneumococcus recovered from the dust of 62 rooms in which pneumonia had not occurred.

From these 62 specimens of dust, in 18 instances, or 29 per cent pneumococcus was recovered. In all but one instance these pneumococci belonged to those types which are normally found in the mouth. The specimen of dust from which the Type I pneumococcus was recovered came from a house where a known carrier of a Type I pneumococcus was visiting. From this study it is evident that pneumococcus can be easily recovered from dust. Furthermore, it appears that the strictly disease-producing types of pneumococcus, Types I and II, are not prevalent in dust when patients and healthy carriers of these types of pneumococcus are absent.

⁴ Netter, L. D., *Compt. rend. Soc. biol.*, 1897, iv, series 10, 538.

⁵ Emmerich, R., *Z. Hyg. u. Infektionskrankh.*, 1894, xvii, 167.

TABLE V.

Incidence of Pneumococcus in Dust from Rooms in Which Pneumonia Had Not Occurred.

Pneumococcus.		Incidence.	
			<i>per cent</i>
Type I.....	1		5.5
" II.....	0		0
" II a.....	0		0
" II b.....	4		22
" II x.....	3		16.6
" III.....	2		11
" IV.....	8		44.4
Pneumococcus present.....	18		
" absent.....	44		
	62		

Types of Pneumococci Obtained from Dust of Households in Which Pneumonia of Type I and Type II Had Occurred.

Table VI shows the incidence and types of pneumococcus found in 183 specimens of dust from households where cases of Type I or Type II pneumonia had occurred.

TABLE VI.

Incidence of Pneumococcus in Dust in the Presence of Pneumonia.

Pneumococcus.		Incidence.	
			<i>per cent</i>
Type I.....	25		33.78
" II.....	23		31.08
" II a.....	0		0
" II b.....	2		2.70
" II x.....	2		2.70
" III.....	2		2.70
" IV.....	20		27.02
Pneumococcus present.....	74		
" absent.....	109		
	183		

From 183 specimens of dust collected where cases of pneumonia due to pneumococcus of Type I or Type II had occurred, 74, or 40 per cent, showed pneumococcus. A Type I pneumococcus was found in 25 instances. In only one instance was a Type I pneumococcus recovered where a Type II pneumonia had occurred. This dust was collected 16 days after the patient had left his home. A Type II pneumococcus was recovered in 23 instances. In only one instance was a Type II pneumococcus found in the dust in the room where there had been a case of pneumonia due to Type I.

From this study it is evident that the highly parasitic pneumococci, Types I and II, are very prevalent in dust where cases of the same type of pneumonia have occurred. As the dust from only a comparatively small area of the floor was swept up, the organisms must have been present in a much larger proportion than these figures indicate.

In Table VII is presented a study of the types of pneumococcus obtained from the homes where a case of Type I or Type II pneumonia had occurred. As a rule, the dust was collected only from the patient's bedroom, but in some homes specimens were also obtained from the living-room and other bedrooms. Of 30 homes where a case of Type I pneumonia had occurred, in 13 instances, or 43 per cent, a Type I pneumococcus was recovered from at least 1 room. In 2 instances a Type I pneumococcus was recovered from 2 rooms. In all, 44 rooms were examined. From 15 of these rooms, or 34.08 per cent, a Type I pneumococcus was recovered. No Type II pneumococcus was recovered from a Type I home. Of 22 homes where a case of Type II pneumonia had occurred, in 13 instances, or 59 per cent, a Type II pneumococcus was discovered in the dust of at least 1 room. In 3 homes a Type II pneumococcus was recovered from 2 rooms. Of the 27 rooms which were examined, in 16, or 59 per cent, pneumococci of Type II were recovered. In only one instance was a Type I pneumococcus recovered from the dust of a Type II house. This occurred 16 days after the patient had left the house.

A comparison of Tables IV and VII shows that in the 28 Type I households in 3 instances the dust was positive, but no contact was found; in 2, the dust was negative, but a positive contact was present;

TABLE VII.

Incidence of Pneumococci in Dust from Homes Where a Case of Pneumonia Occurred.

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
			days			
2,746	I	Patient's bedroom.	0	6	No pneumococci.	Dirty and dark.
2,804	I	" "	3	6	" "	
2,814	I	" "	3	2	" "	Clean, but dark.
		" "	11		" "	
2,815	I	" "	1	4	I	Light, moderately clean.
		" "	12		I	
2,816	I	" "	1	6	I	Dark, fairly clean, poorly ventilated.
		" "	10		No pneumococci.	
2,821	I	" "	1	6	I	
		" "	10		No pneumococci.	
2,824	I	" "	14	3	" "	Very dirty and dark.
2,825	II	" "	1	1	III	Small, dark.
		" "	13		II	
		" "	49		No pneumococci.	
2,827	II	" "	3	3	II	Clean, light, and small.
		" "	10		II	
		" "	19		No pneumococci.	
2,834	II	" "	5	5	IV	Moderately clean, fairly well ventilated.
		" "	16		I	
2,852	I	" "	2	4	No pneumococci.	Large, clean, and light.
		" "	23		" "	
2,853	II	" "	8	2	" "	Very clean, light, well ventilated.
		" "	23		" "	
2,854	II	" "	4	3	" "	
		" "	14		" "	
2,858	I	" "	2	2	I	Moderately clean and light.
		" "	14		IV	
2,868	II	" "	2	4	No pneumococci.	Moderately light, well ventilated.
2,869	II	" "	3	2	" "	Clean.
		" "	18		" "	
2,874	I	" "	3	3	" "	Clean.
		" "	10		" "	
2,879	II	" "	2	2	" "	
		" "	15		" "	

TABLE VII—*Continued.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
			<i>days</i>			
2,880	I	Patient's bedroom.	2	3	IV	Moderately clean,
		" "	13		IV	well ventilated.
2,881	II	" "	2	6	IV	Dark, poorly ven-
		2nd " "	2		IV	tilated.
		2nd " "	13		IV	
2,883	I	Patient's " "	5	4	No pneumococci.	Fairly clean but
		" "	15		" "	small.
2,885	II	" "	3	2	II	Clean.
		" "	13		No pneumococci.	
2,886	II	" "	3	4	II	Fairly clean but
		" "	14		No pneumococci.	dark.
2,890	II	" "	2	4	II	
		" "	15		No pneumococci.	
2,891	I	" "	1	3	" "	Clean.
		" "	23		" "	
2,892	II	" "	1	5	II	Moderately clean,
		" "	23		II	fairly well ven-
		" "	29		No pneumococci.	tilated.
2,896	II	" "	1	4?	II	Fairly clean, well
		" "	24		No pneumococci.	ventilated.
2,901	I	" "	2	5	I	Fairly clean, well
		" "	25		I	ventilated.
		" "	40		I	
		" "	50		I	
		" "	72		No pneumococci.	
		" "	92		" "	
2,906	I	" "	2	2	I	Large, clean.
		" "	21		No pneumococci.	
2,908	I	" "	3	5	I	Fairly clean.
		" "	18		No pneumococci.	
2,913	I	" "	2	5	" "	
		" "	13		" "	
2,917	I	Living-room.	8	6	II x	Fairly light.
		1st sick room.	8		No pneumococci.	Moderately clean.
		1st " "	22		" "	
		2nd " "	8		" "	

TABLE VII—*Continued.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
			<i>days</i>			
2,922	II	Living-room.	3	3	II	Fairly clean, moderately ventilated.
		"	22		No pneumococci.	
		"	37		" "	
		"	70		" "	
		Patient's bedroom.	3		II	
		" "	18		II	
		" "	37		II	
		" "	57		II	Fairly light and ventilated.
		" "	70		No pneumococci.	
2,924	I	" "	3	5	" "	
		" "	21		" "	
		Living-room.	3		" "	
2,925	I	Patient's bedroom.	1	5	" "	
		" "	16		I	
		" "	29		I	Small, dark, moderately clean.
		" "	45		No pneumococci.	
		Living-room.	2		" "	
		" "	29		" "	
		" "	45		" "	
2,926	II	Patient's bedroom.	6	4	" "	
		" "	6		" "	
		" "	20		II	Clean.
		" "	39		II	
		" "	55		No pneumococci.	
2,934	II	" "	5	4	" "	
		" "	14		" "	
2,944	I	1st "	3	5	IV	
		1st "	16		No pneumococci.	House thoroughly cleaned before second dust specimens were collected.
		1st "	53		II x	
		2nd "	3		I	
		2nd "	16		No pneumococci.	
		Mother's room.	3		I	
		" "	16		No pneumococci.	
		" "	53		II x	Dirty, dark, but fairly ventilated.
2,945	I	Living-room.	16		No pneumococci.	
		"	3	5	I	
		"	17		IV	
		"	35		IV	

TABLE VII—*Continued.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
			<i>days</i>			
2,945	I	Patient's bedroom.	3		IV	
		" "	17		IV	
		" "	35		IV	
2,946	II	" "	1	3	No pneumococci.	
		" "	17		" "	
2,949	I	" "	24	8	" "	Fairly clean.
2,952	I	" "	3	2	" "	Light, clean, well ventilated.
		" "	21		" "	
		Living-room.	3		" "	
		" "	21		" "	
2,954	I	" "	5	4	" "	
		" "	19		" "	
		Patient's bedroom.	5		" "	
		" "	19		" "	
2,955	I	" "	4	6	I	Moderately clean.
		" "	13		IV	
		" "	38		No pneumococci.	
		Living-room.	4		" "	
		" "	13		I	
		" "	38		No pneumococci.	
		" "	52		" "	
2,968	I	" "	3	5	IV	Fairly clean, fairly well ventilated.
		" "	19		No pneumococci.	
		Patient's bedroom.	3		" "	
		" "	19		" "	
2,971	II	" "	2	4	II	Well ventilated and clean.
		" "	26		No pneumococci.	
		Living-room.	2		II	
		" "	26		No pneumococci.	
2,976	II	" "	3	3	" "	
		" "	14		" "	
		" "	41		" "	
		Patient's bedroom.	3		" "	
		" "	14		II	
		" "	41		No pneumococci.	
2,984	I	" "	7	5	" "	Dark, dirty basement.
2,991	II	" "	4	1	II	

TABLE VII—*Concluded.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of room.
2,991	II	Patient's bedroom.	26		No pneumococci.	
		Living-room.	4		II	
		"	26		No pneumococci.	
2,996	I	"	1	3	II b	
		"	15		No pneumococci.	
		Patient's bedroom.	1		"	
		"	15		"	
		Another	1		I	
		"	15		I	
M.M.	II	Patient's present room.	0	?	II	
		Patient's present room.	0		No pneumococci.	
F.I.	I	Patient's bedroom.	1	?	I	

Summary.

Type.	No. of pneumonia households examined.	Households giving rise to positive dust.		Total No. of rooms examined.	Positive rooms.	
			<i>per cent</i>			<i>per cent</i>
I.....	30	13	43	44	15	34.08
II.....	22	13	59	27	16	59.25
Total.....	52	26	50	71	31	43.66

in 8, the dust and at least one individual showed a Type I pneumococcus; while in 15, a Type I pneumococcus was recovered neither from the dust nor from a member of the household. In the 22 Type II households, in 10 instances the dust alone was positive; in 1, an individual alone was positive; in 3, a Type II pneumococcus was recovered from both the dust and from a member of the household; and in 8, neither the dust nor an individual showed a Type II pneumococcus. In these 50 households in 13 instances the dust alone

was positive, while in only 3 was the dust negative in the presence of a positive contact.

Individuals or specimens of dust were studied from 54 households where a case of pneumonia due to a pneumococcus of Type I or Type II had occurred. Of the 30 Type I households, 14, or 46 per cent, showed either a positive human contact or a positive dust. From 24 Type II households 15, or 62 per cent, showed either a positive human contact or a positive dust. In other words, at the time of their admission to the hospital, the homes of over 53 per cent of the patients suffering from a pneumonia due to Type I or Type II were infected by a pneumococcus of the same type.

As a rule, the dust became negative before the contacts. But in two instances positive dusts were obtained after the carriers in the households had become negative. It is interesting that in the two homes which showed a positive dust for the longest time lived the two most persistent carriers. In Case 2,901, Type I, the dust was positive for 50 days, while the carrier had not become negative at the end of 80 days. In Case 2,922, Type II, the carrier was still positive at the end of 70 days and the dust was positive on the 57th day.

Two Pneumonia Epidemics.

Through the courtesy of the State Department of Health and the local health officer of Rochester, N. Y., I have had an opportunity of studying two epidemics of pneumonia in institutions.

One epidemic occurred in a boys' asylum. At the time of the epidemic there were over 200 boys in this institution, but the 6 cases of pneumonia were limited to the boys occupying two of the four dormitories. 3 boys in each dormitory developed pneumonia; from the sputum of 3 of the 6 boys a Type I pneumococcus was recovered, from another a Type II x, and from a 5th a Type IV; from 1, no pneumococcus was obtained. The absence of Type I pneumococcus from 3 cases may be due to the fact that this study was not made until the patients were convalescent. The sputum of the other 56 boys who slept in the two dormitories was studied. From the saliva of 6, or 10 per cent, a Type I pneumococcus was isolated.

Three specimens of dust were taken at random from each of the

two dormitories. As these rooms were very clean this dust had to be swept out from between the chinks of the floor boards. One specimen of dust from each dormitory showed a Type I pneumococcus. Of the other four specimens two showed a Type IV, and from two no pneumococcus was obtained. Six specimens of dust from the vacant room used as a ward failed to show a Type I pneumococcus, but a pneumococcus of Type II and a pneumococcus of Type II b were recovered.

In this epidemic 50 per cent of the cases of pneumonia were found to be due to Type I pneumococcus; from 10 per cent of the healthy contacts and from the dust a Type I pneumococcus was recovered.

The other epidemic occurred in the Rochester State Hospital for the Insane. Here six cases of pneumonia occurred among the inmates of one ward of over 200 persons. A Type I pneumococcus was recovered from four of the patients, but the other two patients died before the type of infecting pneumococcus was determined. Each of the patients who died had shared a double room with one of the patients who showed a Type I pneumococcus. A Type I pneumococcus was also recovered from the dust of one of these two bedrooms. The saliva of 148 inmates of this ward was studied. In five instances a Type I, and in one a Type II pneumococcus were isolated. In all, nine specimens of dust were examined. In one instance a Type I, in one, a Type III, and in three, a Type IV pneumococcus was found.

In this epidemic six cases of pneumonia occurred in the ward of an institution. A Type I pneumococcus was recovered from four of the six patients, from 2 per cent of the healthy contacts, and from the dust.

Related Cases of Pneumonia.

Several instances which seem to be contact infections or infections from the same source have been studied. In the first instance a mother daily visited her son who was critically ill with a Type I pneumonia. The mother contracted a bad cold and developed pneumonia due to a Type I pneumococcus 11 days after her son was taken ill. Another case was that of an actor who was admitted to the hospital with a Type II pneumonia. The next day an actor who

shared the same dressing-room developed a Type II pneumonia. Before the end of the week an electrician at the same theater became ill with pneumonia. He also showed a Type II pneumococcus. In a third instance Miss H. (type of pneumonia not determined) was taken to a private hospital by Mrs. A. who developed a Type II pneumonia in a few days. Mr. B. helped to nurse Mrs. A. and shortly fell sick with a Type II pneumonia. In another instance a patient who had just recovered from a Type I pneumonia left the hospital March 9. The next day he went to see his brother who had developed a Type II pneumonia. On March 12 this patient, who had just recovered from a Type I infection, developed a pneumonia due to a Type II pneumococcus. The following case suggests the possibility of an infection by a healthy carrier. A patient was admitted to the hospital suffering from pneumonia due to *Pneumococcus* Type I. Specimens of sputum were obtained from the other members of the household. One 5 year old daughter was found to be a Type I pneumococcus carrier; the other two members of the household were negative. The dust from the room the patient had previously occupied in this house also showed a Type I pneumococcus. The little girl was sent to board with friends while her mother was in the hospital. She spent 3 days with the first family and then went to visit in the Bronx. 6 days after she left, a child in the home where she had visited came down with pneumonia due to *Pneumococcus* Type I. Specimens of sputum from the other members of this household were negative, but from the dust in the sick child's room a pneumococcus of Type I was isolated. The daughter of the original patient visited in the Bronx for 10 days. No cases of pneumonia developed in this home and the sputum of the members of this family as well as the dust failed to show the presence of *Pneumococcus* Type I. The child next went to visit friends in Brooklyn. Although the sputum from the members of this household were negative, from the dust a Type I pneumococcus was recovered.

DISCUSSION.

The results of the work detailed in this paper confirm the previous observations of Dochez and Avery on the occurrence of healthy carriers of disease-producing types of pneumococcus. Consideration

of the results of study over a period of years of the types of pneumococcus inducing lobar pneumonia shows that in the majority of instances infection is due to organisms belonging to Type I or Type II. The minority of cases, on the other hand, are due to infection with pneumococcus of Types III and IV.

Comparison of the types of pneumococcus obtained from the mouth secretions of normal persons with those isolated from individuals with lobar pneumonia shows the existence of two general classes of organisms. One of these, which consists of Types I and II, occurs only in association with disease. The other, which includes Types III and IV and the atypical Type II organisms, also causes pneumonia but these organisms are commonly found in normal healthy mouths. Rarely Types I and II have been found in the mouth secretions of normal individuals who give no history of association with cases of pneumonia. On the other hand, organisms of Types I and II have been found in 11 per cent of normal individuals who have been in intimate association with a case of pneumonia of the same type.

Although the presence of pneumococcus in dust has been known for some time, little significance has been attached to it. The results of this work show that pneumococcus can be easily recovered from dust. The types of pneumococcus found reflect accurately the pneumococcal flora of the mouth of the members of these households. Pneumococcus of Types I and II is rarely found in dust except where a case of pneumonia due to the same type of pneumococcus has occurred. In view of the ease with which dust can be disseminated it is not surprising that in a few instances a Type I or Type II organism was recovered from the dust which did not correspond to the type of pneumococcus producing the disease.

The occurrence of these disease-producing types of pneumococcus in the dust suggests the possibility that air-borne infection may play a part in the production of pneumonia. On the other hand, the mere presence of the disease-producing types of pneumococci in the mouth will not initiate disease. But if a susceptible individual comes in intimate contact with a case of pneumonia there is grave danger of his contracting the disease.

These facts suggest the following conclusions concerning the epidemiology of lobar pneumonia. Infection with pneumococcus of

Types I and II must be regarded as dependent upon either direct or indirect contact with a previous case of lobar pneumonia due to the same type of organism. These types of infection are either acquired by direct contact with a previous case of pneumonia, by association with a healthy carrier of one of these types of pneumococcus, or possibly by an air-borne infection from dust which has been infected. Infection with the sputum types of pneumococcus, namely Types III and IV and the atypical strains of Type II, may be autogenic, or due to the acquisition by the individual of one of these types to which he is especially susceptible.

SUMMARY.

1. Pneumococci of Type I and Type II are responsible for the majority of the cases of lobar pneumonia.

2. Among the pneumococci found in the mouths of healthy individuals Type IV predominates, Type III is frequent, and atypical organisms of Type II are occasionally found.

3. Healthy persons intimately associated with cases of lobar pneumonia may harbor in their mouth secretions the highly parasitic pneumococcus of Types I and II.

4. Occasionally a carrier of Type I or Type II pneumococcus is encountered in whom it is impossible to trace any contact with an infected patient.

5. From the dust of homes where cases of pneumonia due to Types I and II have occurred, pneumococci of the same type may be recovered.

THE PRODUCTION OF ANTIPNEUMOCOCCIC SERUM.

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Ever since the demonstration in 1891 by Foa and Carbone,¹ Emmerich and Fowitzky,² and G. and F. Klemperer³ of the immunizing properties of the serum of animals rendered immune to pneumococcus, this serum, modified in various ways, has been employed to a greater or less extent in the treatment of pneumonia in man.

Besides the employment of sera prepared in various ways in small series of cases, there has been an extensive employment of the sera prepared according to the directions of Pane,⁴ Washbourn,⁵ and more lately Römer⁶ and Neufeld and Händel.^{7, 8} In this country at least six commercial houses which sell biologic products have for some years been producing immune serum for the treatment of pneumonia and pneumococcus infections. It is impossible to know how extensively these sera have been employed, certainly to a sufficient extent to render their production profitable.

In spite of all that has been written concerning the theoretical principles involved in the preparation of antipneumococcic serum, and in spite of all the reports of its therapeutic application which have appeared, it is very difficult to learn from the literature on the subject exactly how these sera have been prepared or standardized.

¹ Foa, P., and Carbone, T., *Gazz. med. Torino*, 1891, xlii, 1.

² Emmerich, R., and Fowitzky, A., *Münch. med. Woch.*, 1891, xxxviii, 554.

³ Klemperer, G., and Klemperer, F., *Berl. klin. Woch.*, 1891, xxviii, 833, 869.

⁴ Pane, N., *Centr. Bakteriöl., 1te Abt.*, 1897, xxi, 664.

⁵ Washbourn, J. W., *Brit. Med. J.*, 1897, i, 510.

⁶ Römer, P., *Arch. Ophth.*, 1902, liv, 99; Experimentelle und klinische Grundlagen für die Serumtherapie der Pneumokokkeninfektion der menschlichen Cornea (Ulcus serpens), Wiesbaden, 1909.

⁷ Neufeld, F., and Händel, *Z. Immunitätsforsch., Orig.*, 1909, iii, 159.

⁸ Neufeld and Händel, *Arb. k. Gsndtsamte.*, 1910, xxxiv, 166, 1293.

Without this knowledge we can have no accurate starting-point from which to proceed toward improvements in methods of production.

In view of these facts and also since an antipneumococcic serum is now being prepared by The Rockefeller Institute for Medical Research and used in the Hospital of this institution, and since serum made in a similar manner is now being prepared by a number of commercial houses and by several public health laboratories for wider distribution, it has seemed important that an accurate description of the methods employed by us in its production be published, together with a brief discussion of the theoretical basis for the methods employed.

*Theoretical Considerations Concerning the Production of
Antipneumococcic Serum.*

In spite of a general belief to the contrary, all kinds of animals, even the most susceptible, may be rendered actively immune to pneumococcus infection, by the previous injection of non-lethal doses of living pneumococci or even by the injection of the dead bodies. Indeed, the substances of the bacteria which give rise to the immune reaction are very resistant to various chemical and physical agents, and a review of the literature shows that it is possible to produce active immunity with a great variety of antigens prepared from the bacteria. The degree of immunity, however, differs, depending upon the procedures employed.

The serum of the actively immunized animals, in many cases, possesses protective and curative power, the degree of this power depending somewhat on the height of the active immunity, but not invariably or regularly so. It may be stated, however, that animals whose serum is protective and curative are always actively immune. On the other hand, an animal may itself be fairly highly immune without the serum containing any immune bodies that we can demonstrate, and without its having any demonstrable protective action. For instance, we have immunized rabbits so that they have successfully withstood 0.1 cc. of a living culture, of which 0.000001 cc. killed the control, without the blood showing a trace of immune bodies or any protective power. Quite frequently animals whose serum shows only moderate grades of immunizing value may be actively resistant

to enormous doses. The fact that active immunity and serum-immunizing power do not run exactly parallel does not necessarily indicate that active immunity in pneumococcus infection differs fundamentally from passive immunity, but suggests this possibility. At any rate, it suggests that in the reaction of resistance to, or recovery from, infection there may be other factors concerned than the humoral ones. Nevertheless, with present knowledge of serum therapy our effort must be confined to the production of a serum having a high content in demonstrable antibodies and a high protective value. The chief kinds of antibodies which can be demonstrated in this immune serum are agglutinins and opsonins or bacteriotropins, and the kind of protective power meant is that which is exhibited when a very small amount of the serum is injected into a susceptible animal such as the mouse, simultaneously with, or within a few hours following, the injection of a large dose of virulent culture, which alone would cause rapid death of the animal. These are the properties of the serum which are at present tested to indicate its therapeutic strength.

It would lead us far from our present purpose, were we to discuss here at length the mode of action of the serum, for the discussion would necessarily involve a consideration of the mechanism of resistance and recovery. It is not believed that the action of the immune serum is entirely dependent either on its power of causing agglutination (Bull⁹) or on its bacteriotropic power (Neufeld and Rimpau¹⁰ and Neufeld and Händel^{7, 8}), though these properties may play important parts. They, however, are susceptible of quantitative estimation. The protection of small animals undoubtedly reproduces more accurately the part which the serum plays in recovery from natural infection, as seen in the human patient, but even here the conditions are not identical.

At the present time, however, the effectiveness of the serum seems to be parallel to its content in the antibodies we have mentioned, and especially to its protective power tested as we have described. We believe the production of antipneumococcic serum that may be accurately standardized is of fundamental importance. Whether all theoretical qualifications are fulfilled is not so essential.

⁹ Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.

¹⁰ Neufeld, F., and Rimpau, W., *Z. Hyg. u. Infektionskrankh.*, 1905, li, 283.

Specificity.

The primary requisite for the serum is that it is specific. Everything stated above concerning both active and passive immunity is true only if the bacterium acted upon, either in antibody tests *in vitro*, or in protection tests *in vivo*, is identical with the organism used in producing the serum. This does not merely mean that the bacteria shall belong to the same species, for it is now generally known that different races of the same species of bacteria may show differences in their antigenic properties, even without differences in their cultural characteristics. These differences have heretofore, however, been considered differences merely in degree and mainly in lesser degree. To overcome the difficulties which this fact puts in the way of the production of immune serum for practical purposes, recourse has been had to the method of immunizing an animal with many different strains, producing a polyvalent serum. This method, for instance, has been made use of to a considerable extent in the production of antimeningococcic serum. Although certain races of meningococci differ from each other quite markedly in their immunological properties, yet they all have common characteristics which render a sharp differentiation difficult, if not impossible. The conditions as regards meningococci at present seem to be somewhat as follows. If we employ the Ehrlich nomenclature, each strain of meningococcus is endowed with a large number of kinds of receptors which we may designate by the letters of the alphabet. In one large group of strains of meningococcus *a* receptors predominate; in another group *b* receptors predominate, etc. In smaller groups other receptors, such as *k*, *l*, *m*, or *n* receptors, are most numerous. However, even in the first two groups, the *a* and *b* receptors are not exclusively present or even overwhelmingly predominating. Indeed each of the different races possesses practically all the different kinds of receptors but in greatly varying degrees.

Under these conditions, in order to produce a serum which will be active against all the different races it is necessary to choose and employ for immunization a large number of races in order that the entire receptor "spectrum" shall be covered as uniformly as possible. It seems that it is possible to do this fairly well as far as meningococcus is concerned.

The conditions as concerns pneumococcus are similar, though not identical. From agglutination experiments Kindborg¹¹ decided that all strains of pneumococci were immunologically distinct. Neufeld first brought evidence of group relationships and this fact has been elaborated and extended by the work in The Rockefeller Institute Hospital and elsewhere. Dochez and Gillespie¹² have demonstrated that the pneumococci obtained from cases of pneumonia occurring in this country belong in four large groups. The immunological characteristics of the organisms of three of these groups are very specific. The conditions therefore among pneumococcus are different from those obtaining among meningococcus. It is difficult to demonstrate by immunological methods that pneumococcus of Type I has any immunological characteristics (or receptors in the Ehrlich nomenclature) which are common to all pneumococci. Complement fixation tests, however, seem to show that the pneumococci of the different types do possess some common characters, at least these tests are not absolutely specific as regards the different types (Hanes¹³) and the antihemolytic reactions of specific sera against the hemolytic toxins derived from different types of pneumococci (Cole¹⁴) are not absolutely specific. So too the extremely active agglutinating immune sera produced by the injection of pneumococci of specific types may have slight degrees of effectiveness against certain strains of other types (Blake¹⁵). But in general the different types of pneumococci have a high degree of specificity as shown by protective action and by agglutination. Active immunity to the different types also seems very specific. This type specificity, which was only lately recognized, is of fundamental and primary importance in considering the practical application of immunity to therapy in this disease. Since nothing was known of this group specificity before the observations of Neufeld were made, it is very difficult to draw any conclusions from the observations or from the results of their practical application which were made in this field before that time. Where identical strains were

¹¹ Kindborg, A., *Z. Hyg. u. Infektionskrankh.*, 1905, li, 197.

¹² Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

¹³ Hanes, F. M., *J. Exp. Med.*, 1914, xix, 38.

¹⁴ Cole, R., *J. Exp. Med.*, 1914, xx, 346.

¹⁵ Blake, F. G., *J. Exp. Med.*, 1917, xxvi, 67.

used throughout experiments, the conclusions of course were valid, but in the absence of knowledge of the importance of using only these strains, it is probable that in many cases this procedure was not strictly adhered to.

The work done in the Hospital of The Rockefeller Institute, both experimentally and clinically, indicates that immune serum against Type I infection is more effective than that against any other of the specific types. Indeed the results obtained both here and elsewhere indicate that this serum has great therapeutic value when it is employed in the treatment of cases due to the same type of pneumococcus. On the other hand, the observations which have so far been made with sera of the other types do not show that these produce distinct therapeutic effects. It is possible, of course, that improvements in the method of production or mode of application of these other types of serum may lead to more satisfactory results. For the present, however, we believe that the general practical application of this form of therapy should be confined to Type I cases, and this may readily be done since the type of infection in the individual case may easily be determined by the methods described elsewhere (Blake¹⁵). We also think it important at present that the commercial production of antipneumococcic serum for therapeutic purposes be confined to that effective against Type I infection. This paper aims to present the best method for manufacturing this serum and is based on the experience we have gained through the immunization of a very large number of rabbits, several goats, and four horses to Type I pneumococcus, four horses to Type II pneumococcus, one to Type III pneumococcus, and one to both Type I and Type II pneumococci, or ten horses in all.

Animals to Be Employed for Immunization Purposes.

For practical reasons small animals cannot be used to supply serum for therapeutic purposes, especially if the large amounts of serum which are now considered necessary are to be used.

In the earlier therapeutic tests, such as those of G. and F. Klemperer and Foa and Carbone, rabbits were used for obtaining the small amounts of serum used. Later Washbourn used ponies for immunizing purposes. Pane used a cow and an ass. Neufeld used horses in the preparation of his serum.

Römer⁶ has laid stress on the importance of combining the sera prepared by immunizing a number of different animals. His reason for this is a purely theoretical one; namely, that all the different antigenic elements contained in a complex structure such as the pneumococcus may not find suitable receptors in any one animal. With present knowledge it seems that we may disregard this consideration.

We believe that for practical reasons it is best to use horses. Our experience is not sufficient to enable us to have a definite opinion as to the kind of horses best to choose for this purpose; apparently, however, draft horses are superior to the lighter grades and to the more finely bred animals. Joint injuries, indeed injuries of all kinds, undoubtedly contribute to the localization of pneumococcus and consequent focal infection. The highly strung, nervous horses are more liable to these injuries and infections. These accidents delay immunization. As regards antibody response, there has been marked individual variation, but the difference has not been associated with any obvious distinction in type of horse. In rabbits, with which our experience is much larger, there is very great individual difference. It is far more important in the production of this kind of serum than in the production of antitoxic sera that the horses should be perfectly sound and healthy, especially without old joint injuries.

Site of Injection.

Neufeld has laid stress on the value of intravenous injections. We have also employed this method since local reactions are thereby avoided. With dead cultures the local reactions are usually not severe or important; where living cultures are employed, however, they may be violent and abscess formation not infrequently occurs. It is felt, moreover, that by intravenous injections the bacteria are brought more rapidly and quickly and with less opportunity for change into contact with widely distributed cells, the reaction with which is supposed to result in the immunity response.

Choice of Antigen.

Use of Living or Dead Cultures.—As we have previously stated, the production of active immunity is attended with little difficulty and the form of antigen, so far as we know, is not of great importance,

but when we come to the question of the production of humoral immunity, especially of the highest grade, this factor may be of the greatest importance. It has generally been assumed on theoretical grounds that to produce the highest grade of humoral immunity it is necessary to use living cultures. It seems likely that all the various constituents of the bacterial cell and even some products of growth, which may be very labile, give rise to specific antibodies. Therefore to obtain the most varied and complete reaction it has been thought necessary to employ the living organisms, which may, for a short time at least, grow in the body and produce or give rise by their dissolution to certain labile metabolic substances which may stimulate antibody formation. Most of the antipneumococcic sera which have been prepared, at least of late years, have been produced by the injection first of dead, then of living organisms. The general acceptance of this method is probably due in part to what is known concerning immunity in tuberculosis. Here, as is well known, the only immunity that is effective has been produced by the injection of living organisms, by producing a mild tuberculous infection. One must be careful, however, in applying what is known concerning one type of infection directly to another, without due consideration. As knowledge of infection increases it becomes more and more evident that each disease must be considered independently. As we shall show later, it is possible that in the production of antipneumococcic serum the living organisms are not so important as they have previously been considered.

Virulence of Organisms Employed.—Pneumococci may rapidly lose their virulence for animals when grown in an artificial medium. This loss of virulence, however, may not be uniform for all kinds of experimental animals. Moreover, the virulence of pneumococci which have long been grown on artificial media may be increased by passing repeatedly through experimental animals. Here again the increase of virulence may not be uniform for all the varieties of animals. It is possible, for instance, to obtain a given strain which is very virulent for guinea pigs, with little virulence for rabbits. Even for animals as closely related in their susceptibility to pneumococcus infection as the rabbit and mouse, it is possible to have races which are highly virulent for one with little virulence for the other.

It is manifest, therefore, that we can judge only very imperfectly of the virulence of a given race for man by determining its virulence for a susceptible animal, such as the mouse or rabbit. Whether in the production of an immune serum it is important for the organisms employed to be highly virulent or not is not known. Neufeld and his associates^{7, 10} have laid stress on the employment of virulent cultures, believing that the action of immune serum is to neutralize exactly those receptors of the bacterium upon which virulence depends. They state that they have proved experimentally that immunity cannot be produced with avirulent races, but give no protocols. Races virulent for mice have been employed in all our immunizing studies and we have no observations concerning immunization with avirulent races. If virulence of the organism used as antigen is important, what we have just stated concerning virulence for different species of animals becomes of great significance. It will be remembered that mice are used in all our tests of immunity. The fact that a serum is protective for mice would indicate that it had been produced by a race of pneumococci virulent for mice, but the test might give us little information relative to its protective power for man. We have therefore come to believe, on these purely theoretical grounds, that the immunization of horses should be carried on with organisms which have not been long under artificial cultivation since their isolation from the human body. These organisms are practically always virulent for mice, so that where they are employed, the test of the protective power of the serum in mice is probably a good test for protective and curative power in man. In order to have a culture which has not long been removed from the human body always ready for use, advantage may be taken of an observation made by Heim¹⁶ and confirmed by Neufeld and Händel⁷ and abundantly corroborated by us; namely, that when pneumococci are preserved in pieces of dried tissue or blood they remain viable for a very long time, and also retain their virulence undiminished. For preserving small amounts of culture the spleens of infected mice, dried and kept in a vacuum, are most satisfactory. For preserving large amounts of culture from human patients, it is well to inoculate a rabbit with blood or other infected material. After infection has reached a maximum grade,

¹⁶ Heim, L., *Z. Hyg. u. Infektionskrankh.*, 1905, 1, 123.

the rabbit is bled and the blood is spread in thin layers in Petri dishes and dried. To obtain a fresh culture at any time all that is necessary is to inoculate a little of this dried material into the peritoneal cavity of a mouse and later make a fresh culture from the heart's blood. For immunizing purposes a fresh culture obtained in this way should be prepared every 1 or 2 weeks. This precaution in obtaining cultures should be taken not only when the live bacteria are to be injected, but also when dead organisms are to be employed.

Method of Growing Organisms Used for Injection.—The use of bacteria grown in broth and separated by centrifugalization from the medium in which they are grown was first employed by Neufeld¹⁷ who believed that the substances formed in the medium during growth are not useful but indeed harmful. We also think that it is not necessary to use the fluid in which the bacteria are cultivated. In this belief we differ from Wadsworth¹⁸ whose observations apparently show that the serum produced with whole cultures is more effective than that produced by the injection of the bacteria alone. His experiments, however, are not entirely conclusive and in the absence of any method of accurately titrating this increased efficacy, it does not seem advisable to employ the whole culture. Moreover, the injection of the whole culture adds greatly to the difficulty of the immunizing process. As is well known, even fresh bouillon is toxic and after bacterial growth has taken place it is still more toxic. In using whole cultures, therefore, one is much restricted as to the amount that can safely be injected. When the amount of culture to be injected reaches a large size, the technical difficulties and time required in centrifugalizing the cultures become considerable. We have attempted to overcome these difficulties by growing the pneumococci on blood glucose agar in flasks and washing off the surface growth in salt solution, using the emulsion so obtained, without centrifugalizing. For certain theoretical reasons, moreover, we thought this method might be of advantage. In our experience, however, the method of growing in bouillon and centrifugalizing still proves the most satisfactory. In our earlier work the organisms were obtained by growing in broth,

¹⁷ Neufeld, F., *Z. Hyg. u. Infektionskrankh.*, 1902, xl, 54.

¹⁸ Wadsworth, A. B., *J. Exp. Med.*, 1912, xvi, 78.

centrifugalizing, and washing once in salt solution, and then re-suspending in salt solution. In our later work, however, we have not thought it necessary to wash, but have merely centrifugalized and then made an emulsion of the sediment in salt solution.

For obtaining a satisfactory growth in bouillon the reaction of the medium is of great importance. Pneumococci grow best in a medium the reaction of which is 0.3 to 0.5 per cent acid to phenolphthalein. If the reaction is more acid than this, a satisfactory growth may not be obtained. We have added no sugar, serum, or other enriching substance to the medium employed.

Size and Spacing of Doses.

In our first studies we employed the method which has been largely employed by others, making the injections every 7 to 8 days. This is the method generally employed in immunization and has developed from the observation that the most efficient stimulus can be applied at the time when the immunological response to the preceding dose is most active. Grades of high immunity have been thought to increase in a step-like manner, each increment being added to that previously present. It is again not certain, however, that anti-pneumococcus immunity obeys the same laws as other forms of immunity in which this step-like rise occurs. Certain of our observations to be mentioned later indicate that even when the injections are made at the period of greatest activity, instead of a rise in the immunity, there may be a fall, especially if the dosage is too large. After a primary immunity had been obtained by weekly injections of dead cultures, live organisms were injected, beginning with small doses, *i.e.*, the bacteria from 2 to 5 cc. of bouillon culture, and the succeeding weekly injections were gradually increased in size up to the bacteria contained in 1 or even 2 liters of culture. Neufeld speaks of injecting doses of living pneumococci as large as the bacteria obtained from 1,500 cc. of culture in the horse, and from 3,500 cc. in the ass. He injected doses of dead organisms as large as the bacteria contained in 9 liters of culture. In our experience this method of immunization is attended with many disadvantages. It has required 6 to 8 months to bring horses up to the desired grade of immunity. That these very large doses are not necessary is shown

by the following protocol of a horse in which the attempt was made to produce an effective serum by using only small doses of culture (Table I).

TABLE I.
Horse 1. Immunized to Pneumococcus Type I.

Date.	Injection.	Tests of serum.
1916		
Feb. 2	3,250 units of tetanus antitoxin subcutaneously.	
" 4	Bacteria from 25 cc. of culture, killed by heating.	
" 11	" " 50 " " " " " "	
" 18	" " 75 " " " " " "	
" 26	" " 100 " " " " " "	
Mar. 3	" living, from 2.5 cc. of culture.	
" 11	" " " 5.0 " " "	
" 19	" " " 10.0 " " "	
" 29	" " " 20.0 " " "	
Apr. 3	" " " 40.0 " " "	
" 15	" " " 80.0 " " "	
" 22		Agglutination:* complete Protection:† 0.1 cc., D.; 0.01 cc. S.
" 22	Bacteria, living, from 120 cc. of culture.	
May 7		Agglutination:* complete. Protection:† 0.1 cc., S.

* These tests were made before we commenced the routine accurate titration of the agglutination strength.

† In the protection tests each of the mice received 0.2 cc. of serum simultaneously with a graduated dose of culture, both given intraperitoneally. The figures given indicate the amount of culture added. D. indicates died; S., survived. For brevity, only the highest dose with which recovery took place is given. In all cases the control animals receiving 0.000001 cc. of culture alone died.

It is true that the results obtained with this horse were unusually good, better than any we have since been able to obtain with a similar method. They indicate, however, that the large doses which we had been using were not necessary, that equally good results could be obtained with much smaller amounts of culture.

Modification and Improvements in the Methods of Immunization.

Over a year ago we undertook experiments to determine whether or not animals could be immunized more rapidly than had been done in the past, and also whether it might not be possible to obtain a higher grade of immunity than we had previously observed, and finally to determine for ourselves whether the use of living organisms is necessary in producing humoral immunity.

To determine the best methods of immunization a large number of rabbits was immunized in various ways and the development of immunity studied. Certain observations which had been made indicated that the process might be hastened by more frequent injections of antigen than had previously been used.

In 1900 Dean¹⁹ showed that in the production of diphtheria antitoxic immunity the administration of the toxin at 3 day intervals gave very successful and practical results. Daily doses of antitoxin have been administered with good results when other methods have failed.²⁰ In 1908 Fornet and Müller²¹ showed that precipitating sera could be produced very rapidly by three daily injections of antigen, bleeding on the 12th day. Bonhoff and Tsuzuki²² confirmed these observations and Tsuzuki²³ showed that by a similar method a rapid production of typhoid-agglutinating serum could be produced. Similar observations have been made by Gay and his assistants.²⁴

Flexner and Amoss²⁵ have employed a similar method in the production of antidysenteric serum, injecting live cultures on 3 successive days, with excellent results. In the same way the method of three daily injections has been employed by Amoss and Wollstein²⁶ in the production of antimeningococcic serum.

In the production of antidysenteric and antimeningococcic serum stress has been laid on regulating the size of the dose so that, following each inoculation, a febrile reaction shall be obtained. It occurred to us that the choice of three daily doses had been made more or

¹⁹ Dean, G., *Tr. Path. Soc. London*, 1900, li, 15.

²⁰ Personal communication from Dr. Theobald Smith.

²¹ Fornet, W., and Müller, M., *Z. biol. Techn. u. Method.*, 1908-09, i, 201.

²² Bonhoff, H., and Tsuzuki, M., *Z. Immunitätsforsch., Orig.*, 1909-10, iv, 180.

²³ Tsuzuki, M., *Z. Immunitätsforsch., Orig.*, 1909-10, iv, 194.

²⁴ Gay, F. P., *Ergebn. Immunitätsforsch. exp. Therap., Bakteriöl. u. Hyg.*, 1914, i, 231.

²⁵ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 515.

²⁶ Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403.

less arbitrarily and that possibly daily doses administered over a longer time might be still more efficacious in producing a satisfactory result, especially since this method had proved of value in the production of diphtheria antitoxin. That this method is indeed of much value is seen from the results of the experiments given below. The experiments need not be described here in detail. Certain slight variations due to external causes were made in individual instances, but most of the animals were treated about as follows.

Series 1.—These animals received weekly intravenous injections of large amounts of bacteria obtained by centrifugalization of broth cultures, which were then killed by heating for 1 hour at 56°C. The size of the doses varied from the bacteria contained in 100 cc. of broth to those contained in 500 cc.

Series 2.—These animals received intravenous injections of very small amounts of bacteria killed by heat as in the above experiment; the doses were given daily for 7 days, then an interval of 7 days was allowed to elapse, and a second series of injections was given, etc. The bacteria in the individual doses varied from those contained in 1 cc. of broth to those contained in 2 cc.

Series 3.—These animals received combined doses of immune horse serum and living cultures intravenously, beginning with 1 cc. of serum plus 0.1 to 0.5 cc. of culture. This dose was repeated every day for 3 days, then 7 days were allowed to elapse, and a second series of these combined doses was given, this time using slightly larger doses of culture. After an interval of another week, combined doses using still larger amounts of culture were given, etc.

Series 4.—These animals received varying sized doses of pneumococci killed by the addition of 0.5 per cent carbolic acid. The bacteria were centrifugalized from broth cultures, taken up in salt solution, the carbolic acid was added, and the mixture kept at 37°C. over night. As in the preceding series, the injections were given intravenously on 3 successive days, then an interval of a week was allowed to elapse, and the second series given. The number of bacteria in the individual doses varied from those contained in 2 cc. of broth to those in 50 cc. of broth. Some animals received large weekly doses subcutaneously.

Series 5.—These animals received injections of an antigen prepared as follows. The bacteria were grown in broth, centrifugalized, washed, and taken up in a very small amount of salt solution, and this emulsion was added to a large amount of acetone. The sediment which formed was centrifugalized at once, dried in a vacuum, and after 12 hours in the vacuum, taken up in salt solution and thoroughly shaken. From this somewhat viscid translucent fluid further dilutions in salt solution were made. The injections in these rabbits were made once a week, and the doses varied from an amount of antigen representing the bacteria contained in 5 cc. of broth to one representing the bacteria contained in 220 cc. of broth. The small doses were given intravenously, the larger ones subcutaneously.

In addition to the above, a small series of rabbits received injections of antigen prepared by freezing and grinding the bacteria, and another small series received injections of bacteria dissolved in bile.

In studies such as these it must be borne in mind that individual rabbits immunized in exactly the same way may show quite marked variations in their immunity response. Slight differences, therefore, in results obtained in small series of animals are not of significance.

It would lead us too far to attempt to analyze in detail the results in the various tests or to publish the protocols. One definite fact stands out from these studies. Uniformly the results following the injection of small doses of killed culture given daily over a period of 7 days followed by 7 days of rest were excellent (Series 2). This is in marked contrast to the results obtained by the injection of large doses of killed cultures given at intervals of a week (Series 1). Serum from these animals showed little or no evidence of immunity. This is strikingly shown in Table II.

TABLE II.

Date.	Day.	Weight.	Injection.	Agglutination tests.	Protection tests.
Rabbit 1.					
1916		gm.			
May 27	1	1,670	Bacteria from 250 cc. of culture, killed by heating.		
	7 day interval.				
June 4	9	1,400	Bacteria from 250 cc. of culture, killed by heating.		
	9 day interval.				
" 14	19	1,300	Bacteria from 250 cc. of culture, killed by heating.		
	9 day interval.				
" 24	29	1,270	Bacteria from 250 cc. of culture, killed by heating.		
	6 day interval.				
July 1	36	1,150		Undiluted, 0	0.001 cc., D.

TABLE II—*Continued.*

Date.	Day.	Weight.	Injection.	Agglutination tests.	Protection tests.
Rabbit 2.					
1916		gm.			
May 28	1	1,520	Bacteria from 1 cc. of culture, killed by heating.		
" 29	2		Bacteria from 1 cc. of culture, killed by heating.		
" 30	3		Bacteria from 1 cc. of culture, killed by heating.		
" 31	4		Bacteria from 1 cc. of culture, killed by heating.		
June 1	5		Bacteria from 1 cc. of culture, killed by heating.		
" 2	6		Bacteria from 1 cc. of culture, killed by heating.		
	7 day interval.				
June 10	14	1,650	Bacteria from 1 cc. of culture, killed by heating.		
" 11	15		Bacteria from 1 cc. of culture, killed by heating.		
" 12	16		Bacteria from 1 cc. of culture, killed by heating.		
" 13	17		Bacteria from 1 cc. of culture, killed by heating.		
" 14	18		Bacteria from 1 cc. of culture, killed by heating.		
" 15	19		Bacteria from 1 cc. of culture, killed by heating.		
	7 day interval.				
June 23	27	1,180	Bacteria from 1 cc. of culture, killed by heating.		
" 24	28		Bacteria from 1 cc. of culture, killed by heating.		
" 25	29		Bacteria from 1 cc. of culture, killed by heating.		
" 26	30		Bacteria from 1 cc. of culture, killed by heating.		
" 27	31		Bacteria from 1 cc. of culture, killed by heating.		
	8 day interval.				
July 6	40	1,100		1:200 + 1:400 0	0.2 cc., D 0.1 cc. S.

As the protocols show the serum of Rabbit 1 injected weekly with large doses of culture showed no agglutinating power and only moderate protective action. On the other hand, the serum of Rabbit 2 which was treated over practically the same period of time but received very small daily doses showed high agglutinating strength, positive in a dilution of 1:200, and high protective power. These results were most surprising and striking, especially as all of the eight rabbits inoculated with the small daily doses showed an extraordinarily prompt and active response, while all of the eight rabbits inoculated with the large doses showed very little or no response in the same period of time.

The studies of other methods of immunization gave little information. The attempts to produce immunity with combined doses of serum and culture (Series 3) gave unsatisfactory results. The experiments of this series were undertaken because by this method live cultures could be injected at the very beginning of the immunization. Moreover, Theobald Smith²⁷ and von Behring²⁸ have shown the possibility of immunizing with combined doses of toxin and antitoxin. Besredka²⁹ and others have shown the possibility of immunizing against typhoid with sensitized cultures. Levy and Aoki³⁰ claim to have produced immunity to pneumococci with great rapidity (in 6 hours) by the injection of sensitized bacteria killed with carbolic acid. However, in view of what is now known concerning possible dissociation of pneumococcus antigen and antibody (Gay and Chickering³¹), it is possible that what the latter writers observed was not active immunity but slight grades of passive immunity. In any case, the grade of immunity produced was slight. Our experiments yielded no evidence in favor of the combined injection of culture and immune serum. Nevertheless, it is possible that other modifications of the method might yield better results, especially as we made no effort to balance accurately the amounts of culture and immune serum employed.

²⁷ Smith, Theobald, *J. Med. Research*, 1907, xvi, 359.

²⁸ von Behring, E., *Deutsch. med. Woch.*, 1913, xxxix, 873.

²⁹ Besredka, cited by Gay.²⁴

³⁰ Levy, E., and Aoki, K., *Z. Immunitätsforsch., Orig.*, 1910, vii, 435.

³¹ Gay, F. P., and Chickering, H. T., *J. Exp. Med.*, 1915, xxi, 389.

The serum of the rabbits of Series 4 inoculated with cultures killed by carbolic acid indicated no considerable grade of immunity. There is apparently no advantage to be gained in employing antigen prepared in this way. Levy and Aoki,³⁰ however, have reported the production of immune serum in dogs by the use of this method; but the grade of immunity, according to our standards, was slight.

Finally, the experiments in Series 5 and those in which frozen and ground bacteria and bacteria dissolved in bile were employed simply offer observations in the use of bacterial antigenic substances produced in other ways.

Neufeld³² and Vetrano³³ have also employed bile extracts of pneumococci for immunizing purposes, only, however, in the production of active immunity. Others have employed still other artificial methods of treating the bacteria. G. and F. Klemperer³ used glycerol extracts and Wadsworth¹⁸ attempted immunization with cultures precipitated with alcohol and dissolved in water.

These and our own observations with artificially produced antigens show that slight grades of immunity can be produced by antigens prepared by various methods. The antigenic substance seems to be highly resistant. However, neither the observations of others nor our own indicate that these methods are especially useful in extracting the antigenic substance or in rendering it more effective. Indeed in all instances the immunity reaction resulting from the employment of antigens prepared in these ways was less intense than that following the injection of heat-killed bacteria. One fact emerges from these experiments, however, though in a less striking way than from the observations previously mentioned; namely, that small doses repeated frequently are much more effective than large doses given at longer intervals. The very large doses seem to have a definite repressing action on the development of antibodies.

These observations led us to immunize a series of four rabbits with small daily doses to determine the exact time of appearance of the immune properties in the serum. Table III is a typical protocol of one of the rabbits.

³² Neufeld, Z. *Hyg. u. Infektionskrankh.*, 1900, xxxiv, 454.

³³ Vetrano, G., *Centr. Bakteriolog., 1te Abt., Orig.*, 1909, lii, 275.

TABLE III.

Rabbit 3.

Date.	Weight.	Injection of vaccine.	Agglutination tests.	Protection tests.
1916	gm.	cc.		
May 29	1,950		0	0.0001 cc., D. 36 hrs. 0.00001 " " 36 " 0.000001 " " 36 "
" 29	1,950	1*		
" 30		1		
" 31	1,900	1		
June 1		1		
" 2		1		
" 3		1		
" 9	1,910		1:1 ++ 1:10 0	0.1 cc., D. 16 hrs. 0.01 " " 79 " 0.001 " S. 0.0001 " "
" 11		1		
" 12		1		
" 13	1,900	1		
" 14		1		
" 15	1,750	1		
" 16		1		
" 23			1:1 ++ 1:10 ++ 1:20 +	0.1 cc., S. 0.01 " " 0.001 " "
" 24		1		
" 25	1,750	1		
" 26		1		
" 27	1,900	1		
" 28		1		
" 29	1,850	1		
July 5	1,950			
" 7			1:100 ++ 1:200 + 1:400 0	0.2 cc., D. 20 hrs. 0.1 " S. 0.01 " "

* In these experiments the material for injection was prepared at the beginning of the experiment and kept on ice. 150 cc. of an 18 hour broth culture of *Pneumococcus* Type I was centrifugalized and the sediment washed once in sal solution. The sediment was taken up in 10 cc. of salt solution and heated $\frac{3}{4}$ hour at 56°C. Cultures were sterile. The emulsion was kept on ice and, after shaking, a small amount was removed and diluted to original volume before each injection. The injections were made intravenously.

The experiment shows that it is possible by this method of immunization to produce a high grade of immunity within 6 weeks; even within 4 weeks a considerable grade of immunity is present. The serum from each of the four rabbits showed a high agglutinating titer and a constant protective power against 0.01 to 0.1 cc. of culture. Two of the rabbits lost slightly in weight, one remained stationary, and one actually gained. They showed no other ill effects.

Experiments have also been made to show whether similar or better results could be obtained by injecting animals daily with small doses over longer periods than 7 days. Four rabbits were given small daily doses for 14 days and then bled and the serum was tested on the 5th and 10th days following the last injection. The serum of these

TABLE IV.

Horse 2. Immunized to Pneumococcus Type I.

Date.	Injection.								Agglutination test.	Protection tests.
1916										
Jan. 27	1,500 units of tetanus antitoxin subcutaneously.									
" 29	Bacteria from 50 cc. of culture, killed by heating.									
" 30	"	"	50	"	"	"	"	"		
" 31	"	"	50	"	"	"	"	"		
Feb. 1	"	"	50	"	"	"	"	"		
" 2	"	"	50	"	"	"	"	"		
" 3	"	"	50	"	"	"	"	"		
" 10	"	"	50	"	"	"	"	"		
" 11	"	"	50	"	"	"	"	"		
" 12	"	"	50	"	"	"	"	"		
" 13	"	"	50	"	"	"	"	"		
" 14	"	"	50	"	"	"	"	"		
" 15	"	"	50	"	"	"	"	"		
" 23	"	"	50	"	"	"	"	"		
" 24	"	"	50	"	"	"	"	"		
" 25	"	"	50	"	"	"	"	"		
" 26	"	"	50	"	"	"	"	"		
" 27	"	"	50	"	"	"	"	"		
" 28	"	"	50	"	"	"	"	"		
Mar. 8								1:200 +	0.1 cc., S. 0.1 " D. 0.01 " S.	

animals has shown very slight agglutinating and protective power, so that this modification seems to be of no advantage.

The results of the rabbit experiments have led us to try the method of immunization described above in horses. The result obtained in one horse treated in this manner is given in Table IV.

The results in this and other horses have shown that it is possible in this way to produce a very high grade of primary immunity with a great saving in time and without danger to the animal. The dosage has been arbitrarily chosen. It is possible that with further experience and more carefully regulated dosage it may be possible to produce even higher grades of immunity in this way. It may even be possible by this method to obviate entirely the use of live cultures.

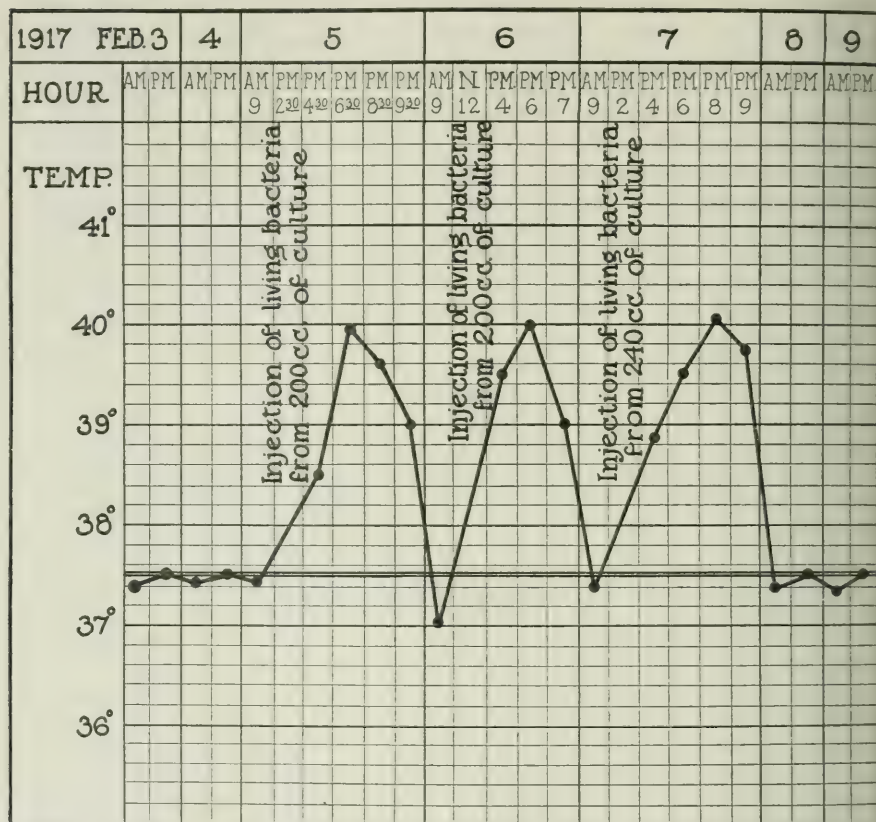
What has been attempted so far, however, has been the production of a primary immunity in the quickest possible time and with the least loss in horses.

TABLE V.

Horse 2, Undergoing Immunization Since January 29, 1916. Last Bleeding, 11 Liters, on May 15, 1917.

Date.	Injection.	Protection tests.
<i>1917</i>		
May 26	Bacteria from 100 cc. of culture, killed by heating.	
" 27	" " 100 " " " " " "	
" 28	" " 100 " " " " " "	
" 29	" " 100 " " " " " "	
" 30	" " 100 " " " " " "	
June 1	" " 100 " " " " " "	
" 9	" " 100 " " " " " "	
" 11	" " 100 " " " " " "	
" 14	" " 100 " " " " " "	
" 15	" " 100 " " " " " "	
" 16	" " 100 " " " " " "	
" 17	" " 100 " " " " " "	
June 25	Blood test.	0.01 cc., D.
July 6	Bacteria, living, from 100 cc. of culture.	
" 7	" " " 130 " " "	
" 8	" " " 160 " " "	
" 13	Blood test.	0.2 cc., S.

The problem now confronting us is to determine the method by which horses having an established primary immunity may be brought up to the highest possible level and kept there. In order to do this, when dead cultures have failed, it may be necessary to resort to living



TEXT-FIG. 1. Temperature curve of Horse 3 injected with living *Pneumococcus* Type I.

cultures. An observation on one of our horses makes this probable (Table V).

In this horse, whereas repeated small daily doses of killed culture failed to bring the serum up to full strength, three moderate sized doses of living culture caused the serum to acquire maximum power.

This result has been seen on numerous occasions and it is probable that in most horses living cultures must finally be employed. When injections of living cultures are made we now employ the method described by Flexner and Amoss,²⁵ administering three doses in amounts sufficient to produce a moderate febrile reaction. A typical curve is shown in Text-fig. 1. So far as pneumococcus immunity, however, is concerned, the necessity for producing febrile reactions is not established. Neufeld believed that so far as agglutinating sera are concerned the power depends not so much on the height of the immunity as on the intensity of the last reaction through which the animal had passed. Therefore he thought it advisable to inject as large amounts as possible without killing the animal. The observations we have made do not support this point of view. In our experience with horses a violent reaction is not always followed by a marked immunity response or increase in agglutinating power of the serum; indeed the opposite is frequently the case. For the present, however, in giving live cultures it is probably better to be guided in the size of the dose by the febrile reactions. We think, however, that large doses should be avoided even though the febrile reaction is slight.

Typical Method of Immunization Based on Previous Observations.

As a result of our observations and those of others we now believe that immunization should be carried out according to the method as at present employed by us, which is briefly given below.

Having obtained a sound, fairly heavy horse, it is first given a glanders test. At present the complement fixation test is used for this purpose. A specimen of 20 cc. or more of blood is obtained before any treatment is given and is kept for use in control tests. The immunization is then carried out as follows. All injections are made intravenously, employing for this purpose a Luer syringe. To avoid any accidental injury to the vein it is well to have the needle attached to the syringe with a small piece of rubber tubing. The culture used for injection, whether living or dead, should be one highly virulent for mice, 0.000001 cc. killing regularly, and it should have gone through very few passages in animals or on artificial media since removal from the human patient. The method for keeping the cultures is described above (page 447). In preparing the material for injection, both living and dead, the organisms are grown on beef peptone broth, reaction 0.3 to 0.5 per cent acid to phenolphthalein. Cultures about 12

to 15 hours old are preferable as at this time maximum growth is present, with a minimum of autolysis (Chesney³⁴). The cultures should contain about 200 to 300 million bacteria per cc. The culture is centrifugalized until the supernatant fluid is clear. With the large centrifuge employed by us this requires about 20 to 30 minutes. The supernatant fluid is then poured off and the sediment is taken up in a small amount of sterile salt solution.

If the organisms are to be injected alive, the emulsion in salt solution is not made until just before injection, so that autolysis and death may not take place. If the organisms are to be injected dead, the emulsion is placed in a tube in a water bath and kept at 56°C. for $\frac{3}{4}$ hour. For the daily injections a considerable amount of the emulsion, after killing, is prepared and kept on ice. This may be employed for all the injections in the series of 6 to 7 days. We think, however, a fresh emulsion should be prepared each week. For each injection the dilution of the fluid should be such that the volume injected is about 20 cc.

The following course of injections is now carried out. Every day for 6 days an amount of the emulsion of killed pneumococci containing the bacteria from 50 cc. of the bouillon culture is injected. An interval of 7 days is allowed to elapse and then a second series of daily injections, of the same size, is made. Again an interval is allowed to elapse and on the 6th day a specimen of blood is obtained for testing. Tests are made at once for agglutinating and protective power. This requires several days.

If the serum causes agglutination in a dilution of 1:200 and is of standard protective value, 0.2 cc. regularly protecting a mouse against 0.1 cc. of a virulent culture, bleeding may be carried out at once; that is, on the 10th to 12th day following the last injection. As a matter of fact, we have never seen the titer of the serum after this amount of treatment to be so high. Consequently it has been our practice, and we advise, that 8 to 10 days after the last injection of the second series of dead bacteria, injection of live organisms be commenced. These injections are given on successive days. The first injection should consist of the bacteria contained in 20 cc. of the original culture. The temperature is taken every 2 hours for 8 to 10 hours following each of the injections of live cultures. If the temperature reaction is only moderate, not over 40.5°C., an injection of the bacteria from 40 cc. of culture is given on the following day. If the reaction from this is only moderate, the dose is again doubled on the following day and the bacteria from 80 cc. of culture are injected. As before stated, so far as pneumococci are concerned it is difficult to regulate the dosage entirely by the febrile reaction obtained. If the reaction is very severe, of course the dose is made smaller than those mentioned. On the other hand, even though the reaction is very slight, we do not advise giving more than the amounts stated. 6 days after the last injection another specimen of blood is obtained for testing. If the serum is of the standard strength, bleeding can now be done. If it is still too weak, a second

³⁴ Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

series of injections of living bacteria is made. These injections should consist of the bacteria from 100 cc., 150 cc., and 200 cc. of broth on the 3 days respectively. Again, the size of the doses may need to be somewhat modified, on account of the severity or lack of febrile reaction. Certain horses may require still further injections of live cultures but this is exceptional. In any case, we think the injections should be made in series of three, given on successive days, with 7 day intervals between each series, and from our present standpoint we believe that the size of the injection should never be greater than the bacteria from 300 to 400 cc. of broth. Following the bleeding, it is well to allow the horse to remain quiet for 3 or 4 days. Then a series of three injections of living cultures, 50, 80, and 100 cc., is again given. After a week the serum is again tested and if of standard strength bleeding may again be done on the 10th day following the last injection.

CONCLUSIONS.

In the production of immune serum for therapeutic purposes strict attention must be paid to the immunological specificity of the bacteria used for immunization. At present the only serum of which the therapeutic value has been proven is that effective against Type I pneumococcus infection. This serum should have agglutinating power for Type I pneumococcus and should have the power of protecting mice against large amounts of virulent culture. Experiments have shown that for producing the primary immunity most rapidly several series of small doses of dead cultures should be given, the injections being made daily for 6 to 7 days, followed by a week in which no injections are made. To produce the highest type of immunity probably living organisms are required. These should be given in moderate doses daily for 3 days, with an interval of a week between each series of injections. By following accurately the methods described, horses may be made to produce rapidly a high grade of specific serum. The observations so far made indicate the importance of employing small doses of culture frequently repeated in this form of immunization.

STUDIES ON ANTIBLASTIC IMMUNITY.*

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The following studies were undertaken with the purpose of throwing more light, if possible, on the influence exerted by immune serum on the metabolic activities of bacteria. It has been shown that under certain experimental conditions immune serum possesses the property of inhibiting or markedly retarding the metabolic functions of various microorganisms. Bacterial metabolic processes are generally considered to be enzymotic in nature. It is not known whether they take place primarily at the surface of the bacterial cell or within the cell body. It is probable, however, that exogenous metabolism occurs to a considerable extent in preparing nutrient material for absorption.

Evidence for this may be found in the studies by Cole¹ on the formation of methemoglobin by the pneumococcus. The property of immune serum which enables it to inhibit the metabolic activities of bacteria is considered to act at the surface of the bacterial cell and to be antienzymotic in nature. The term antiblastic immunity has been applied as descriptive of the phenomenon.

Von Dungern² has shown that the liquefaction of gelatin by *Staphylococcus pyogenes aureus* is inhibited by antistaphylococcus immune serum; Ghéorghiewsky³ showed that immune serum inhibits pigment formation by *B. pyocyaneus*; and Ascoli⁴ considered that the action of antianthrax serum was in part dependent upon the inhibition of capsule formation by *B. anthracis* with consequent suppression of the metabolic activities of the organism. More recently Dochez and Avery⁵ have shown that antipneumococcus immune serum retards the growth of the pneumococcus and inhibits or markedly delays the fermentation of inulin and the splitting of protein by the pneumococcus.

* This work was done under the tenure of a William O. Moseley Travelling Fellowship from Harvard University.

¹ Cole, R., *J. Exp. Med.*, 1914, xx, 363.

² von Dungern, *Centr. Bakteriöl., 1te Abt.*, 1898, xxiv, 710.

³ Ghéorghiewsky, *Ann. Inst. Pasteur*, 1899, xiii, 298.

⁴ Ascoli, A., *Centr. Bakteriöl., 1te Abt., Orig.*, 1908, xlvi, 178.

⁵ Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1916, xxiii, 61.

EXPERIMENTAL.

The majority of the experiments reported below deal with the antiblastic properties of antipneumococcus serum. A few experiments have been done with antistaphylococcus serum.

Inhibition of the Metabolic Activities of the Pneumococcus by Antipneumococcus Immune Serum.

Characteristic metabolic functions of the pneumococcus are the fermentation of inulin and litmus milk, the splitting of protein, and the transformation of oxyhemoglobin to methemoglobin. The influence which immune serum exerts on these functions of the pneumococcus has been studied. The experiments of Dochez and Avery have been repeated and extended and their experimental results confirmed in that under certain conditions antipneumococcus serum has been found to possess the property of inhibiting or retarding the metabolic activities of pneumococci. The conclusions drawn from our experiments as to the mechanism of this inhibitory property of antipneumococcus serum, however, differ from those of Dochez and Avery.

Experiment 1. Inhibition of the Growth of the Pneumococcus by Antipneumococcus Serum.—In Table I is shown one of a series of experiments on the rate of growth of the pneumococcus in homologous and heterologous antipneumococcus serum and in normal horse serum. The amount of growth was determined by the plate method.

This experiment shows that an apparent inhibition of growth occurs in homologous antipneumococcus serum as compared with that in normal serum during a period of 3 hours, that a retardation of growth occurs up to 6 hours, but that by 24 hours an abundant growth is present in the immune serum as well as in the normal serum. It furthermore appears that there is some retardation of growth of Type I pneumococcus by the heterologous immune serum, a phenomenon which does not occur with Type II pneumococcus. These results agree with those of Dochez and Avery. It is felt, however, that the interpretation of such results as indicative of actual retardation of growth is open to serious objection. It is clear that the apparent inhibition of growth by homologous serum must in large part

TABLE I.

Inhibition of the Growth of the Pneumococcus by Antipneumococcus Serum.

Culture 0.00000001 cc.	Serum 0.2 cc.	No. of colonies.			
		Imme- diately.	After 3 hrs.	After 6 hrs.	After 24 hrs.
Pneumococcus Type I	Antipneumococcus Serum Type I	61	54	80	Confluent.
" " I	Antipneumococcus Serum Type II	50	45	12,000	"
" " I	Normal horse serum.	37	284	22,000	"
Pneumococcus Type II	Antipneumococcus Serum Type I	31	69	6,200	Confluent.
" " II	Antipneumococcus Serum Type II	43	42	1,860	"
" " II	Normal horse serum.	29	59	7,750	"

be due to agglutination. Furthermore, it is well known that during the first hours of growth of the pneumococcus chain formation occurs to a considerable extent. This is a variable phenomenon which is difficult to control. For these reasons it seems hazardous to conclude from the experiments that immune serum actually retards the growth of the pneumococcus and quite as probable that the inhibition indicated by the colony counts is more apparent than real.

Experiment 2. Inhibition of the Fermentation of Inulin and Litmus Milk by Antipneumococcus Serum.—The power of antipneumococcus serum to inhibit the fermentation of inulin and litmus milk by the pneumococcus has been tested in a considerable series of experiments, one of which is shown in Table II.

This experiment demonstrates that the fermentation of litmus milk by the pneumococcus is markedly delayed in the presence of the homologous immune serum. The fermentation of inulin is retarded by antipneumococcus serum in a similar manner. In the tubes containing the homologous immune serum the organisms grow in agglutinated masses at the bottom of the tube, while in the tubes containing the heterologous or normal serum they grow diffusely. To demonstrate clearly inhibition of fermentation it was found necessary to use extremely small amounts of pneumococcus culture (not more than 0.00001 cc.) in inoculating the media. With the use of

TABLE II.

Inhibition of the Fermentation of Litmus Milk by Antipneumococcus Serum.

Litmus milk 4 cc. +		Incubation at 37°C.			
Culture 0.000001 cc.	Serum 1 cc.	24 hrs.	48 hrs.	72 hrs.	5 days.
Pneumococcus Type I	Antipneumococcus Serum Type I	—	—	±	+
“ “ I	“ “ II	++	++	++	++
“ “ I	Normal horse serum.	++	++	++	++
Pneumococcus Type II	Antipneumococcus Serum Type I	++	++	++	++
“ “ II	“ “ II	—	+	±	+
“ “ II	Normal horse serum.	++	++	++	++

++ indicates complete acidification and coagulation; ++, acid and incomplete coagulation; +, acid and beginning coagulation; ±, acid and no coagulation; ±, slight acidification and no coagulation; —, no acidification or coagulation.

larger amounts retardation of fermentation was very slight or did not occur at all. In a series of experiments to determine the maximum amount of culture that could be used satisfactorily in demonstrating the antiblastic action of immune serum in preventing the fermentation of inulin, it was frequently noted that in the immediate vicinity of the agglutinated pneumococci at the bottom of the culture tube there was acidification of the medium indicated by change of the litmus indicator to red, while the upper portion of the medium remained unchanged. This observation suggested the possibility that the apparent antiblastic action of antipneumococcus serum was in reality due merely to agglutination of the organisms at the bottom of the culture tube and their consequent inability to come into intimate contact with the medium as a whole, this not being the case in the presence of heterologous immune or normal serum. If this is so, to conclude from such experiments as those cited above that immune serum possesses a specific antiblastic action would hardly be justified. Further experiments on this point are given below.

Experiment 3. Inhibition of Methemoglobin Formation by Antipneumococcus Serum.—The transformation of oxyhemoglobin to methemoglobin is a characteristic metabolic function of the pneumococcus which lends itself readily to *in vitro* experiments. A large series of experiments has been done in order to determine under what conditions antipneumococcus serum will inhibit this

activity of the pneumococcus. In general, the results have been similar to those obtained in the case of litmus milk and inulin. To demonstrate inhibition, it was found essential to use very small amounts of pneumococci in inoculating the hemoglobin solution-serum mixtures and to allow the cultures to incubate 24 hours. One of these experiments is shown in Table III, which demonstrates that under these conditions homologous antipneumococcus serum inhibits the transformation of oxyhemoglobin to methemoglobin by the pneumococcus. Heterologous immune serum and normal horse serum possessed no inhibitory properties.

TABLE III.

Inhibition of Methemoglobin Formation by Antipneumococcus Serum.

Hemoglobin solution* 1 cc. +		Methemoglobin formation.	
Culture 0.00001 cc.	Serum 1 cc.	After 24 hrs.	After 48 hrs.
Pneumococcus Type I	Antipneumococcus Serum Type I	—	—
“ “ I	“ “ “ II	++	++
“ “ I	Normal horse serum.	++	++
Pneumococcus Type II	Antipneumococcus Serum Type I	++	++
“ “ II	“ “ “ II	—	±
“ “ II	Normal horse serum.	++	++

++ indicates complete transformation of oxyhemoglobin to methemoglobin; ±, slight methemoglobin formation; —, no methemoglobin formation.

* Hemoglobin solution = 2 cc. of a 5 per cent suspension of washed rabbit corpuscles + 8 cc. of distilled water + 30 cc. of 0.85 per cent salt solution.

With the use of larger amounts of culture it was found that the formation of methemoglobin was not inhibited by immune serum, but took place rapidly in the immediate vicinity of the agglutinated pneumococci and gradually diffused upward throughout the whole medium. These results, as in the case of inulin, suggested that the apparent inhibition which occurs when minute amounts of culture are used is due to inability of the agglutinated pneumococci to come into intimate contact with the medium as a whole.

Experiment 4. Inhibition of the Proteolytic Functions of the Pneumococcus by Antipneumococcus Serum.—The growth of pneumococcus in a serum broth medium is attended by an increase in the amino-acid content of the medium. Dochez and Avery⁵ believe it probable that the pneumococcus effects a splitting of pro-

tein before absorption and that the increase in amino-acid content of the medium represents the excess of protein split over that used up in the process of growth. If this hypothesis is accepted, the increase in amino-acid produced by the growth of the pneumococcus might serve as a measure of the proteolytic activity of the pneumococcus in a given culture, the amount of increase being directly proportional to the total amount split. The possibility should be pointed out, however, that the amount of amino-acid increase might be inversely proportional to the metabolic activities of the pneumococcus, a small amino-acid increase indicating a greater utilization of the total amount of protein split, and *vice versa*.

The experiment recorded in Table IV shows the increase in amino-acid nitrogen when the pneumococcus was grown in broth containing antipneumococcus serum as compared with the increase in the presence of normal horse serum. The increase in amino-acid was determined by the method of Van Slyke.⁶

TABLE IV.

Inhibition of the Proteolytic Activities of the Pneumococcus by Antipneumococcus Serum.

Broth 8 cc. +		Increase in amino nitrogen per cc. after 48 hrs. at 37° C.
Culture 0.000002 cc.	Serum 2 cc.	
Pneumococcus Type I	Antipneumococcus Serum Type I	mg 0.031
“ “ I	“ “ “ II	0.103
“ “ I	Normal horse serum.	0.106

If the increase in amino nitrogen content of the medium is a direct measure of the proteolytic activities of the pneumococcus, it is apparent that the homologous antipneumococcus serum exerted a marked inhibitory action on this metabolic function of the pneumococcus. Since the pneumococcus growth in the tube containing homologous immune serum was in agglutinated masses at the bottom of the tube in contradistinction to the diffuse growth in the other tubes, it seemed possible that this factor might be responsible for the apparent inhibition of proteolytic activity rather than any true antienzymotic property of the serum. A further experiment to determine the influence of this factor is presented below.

Experiment 5. Antiblastic Properties of Exhausted Immune Serum.—A series of experiments was done to determine whether immune serum exhausted of its

⁶ Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 121; 1915, xxiii, 407.

agglutinin and precipitin content by repeated exposure to killed pneumococci still retained any demonstrable antiblastic properties. In Table V is shown one of these experiments from which it will be seen that immune serum exhausted of its agglutinin and precipitin content possesses no power to inhibit the formation of methemoglobin by the pneumococcus. Identical results have been obtained with respect to the fermentation of inulin and litmus milk. From these experiments it is evident that the specific antiblastic action of immune serum, if such exists, is removed from the serum at the same time that the agglutinins and precipitins are removed by treatment with dead pneumococci.

TABLE V.

Antiblastic Properties of Exhausted Antipneumococcus Serum.

Hemoglobin solution 1 cc. +		Methemoglobin formation.	
Culture 0.00001 cc.	Serum 1 cc.	After 24 hrs.	After 48 hrs.
Pneumococcus Type I	Antipneumococcus Serum Type I	—	—
“ “ I	Exhausted “ “ “ I	++	++
Pneumococcus Type II	Antipneumococcus Serum Type II	—	±
“ “ II	Exhausted “ “ “ II	++	++

Experiment 6. Relation of Agglutinins to Antiblastic Immunity.—From the results obtained in the foregoing experiments the suggestion arose that the apparent antiblastic action of antipneumococcus serum was in some way closely related to agglutination of the bacteria. The following experiment was done to determine more accurately this relationship. Equal parts of pneumococcus cultures and increasing serum dilutions were mixed in small tubes and incubated for 2 hours at 37°C. 1 cc. of hemoglobin solution was then carefully added to each tube so as not to disturb the agglutinated pneumococci and the tubes were incubated for 1 hour at 37°C. The results are shown in Table VI.

This experiment shows that the antiblastic action of the serum bears a definite relationship to the degree of agglutination of the pneumococci. In the tube containing the 1:25 serum dilution agglutination was complete, the pneumococci having entirely settled to the bottom of the tube, and complete inhibition of methemoglobin formation occurred. With increasing dilutions of serum agglutination and sedimentation were progressively less complete and formation of methemoglobin rapidly took place in the immediate neighborhood of the agglutinated pneumococci, the upper portion of the medium

TABLE VI.
Relation of Antiblastic Immunity to Agglutination.

Culture 0.5 cc.	Serum 0.5 cc.	Agglutination after 2 hrs. at 37° C.		Methemoglobin formation after 1 hr at 37° C
Pneumococcus Type I	Antipneumococcus Serum Type I (1: 25)	++	1 cc. of hem- oglobin solution added to each tube.	—
“ “ I	Antipneumococcus Serum Type I (1: 50)	++		—
“ “ I	Antipneumococcus Serum Type I (1: 100)	+		++
“ “ I	Antipneumococcus Serum Type I (1: 200)	±		—
“ “ I	Antipneumococcus Serum Type I (1: 400)	—		++
“ “ I	Antipneumococcus Serum Type II (1: 25)	—		++
“ “ I	Normal horse serum (1: 25)	—		++

— indicates methemoglobin formation in the immediate vicinity of the agglutinated pneumococci, the upper portion of the medium remaining unchanged.

from which the bacteria had settled out remaining unchanged. This experiment indicates that the antiblastic property of antipneumococcus serum probably depends in large measure, if not entirely, upon its ability to agglutinate the pneumococci and thereby prevent their coming into intimate contact with the medium as a whole.

By growing pneumococci in a serum-hemoglobin solution agar medium, agglutination of the pneumococci may be prevented. Under these conditions antipneumococcus serum was found not to inhibit methemoglobin formation. An objection may be raised to these experiments, however, because it is not certain that in a solid medium the immune serum is in sufficient concentration in the immediate vicinity of the pneumococcus colonies to exert a demonstrable antiblastic action.

Experiments 7 and 8. Relation of Antiblastic Immunity to the Ability of Bacteria to Come into Intimate Contact with the Medium.—It seemed probable from many of the previous experiments that the antiblastic action of antipneumococcus serum was due to inability of the agglutinated bacteria to come into sufficiently

intimate contact with the medium as a whole to enable them to produce the characteristic changes in the medium. Under these conditions the metabolic activities of the pneumococci could exert their influence only in the immediate vicinity of the agglutinated bacteria and complete change of the medium could take place only as gradual diffusion occurred. This is exactly what did occur when moderately large amounts of pneumococci were used, the reaction in the presence of homologous immune serum being merely retarded rather than completely inhibited. When minute amounts of pneumococci were used growth had probably ceased before diffusion could take place and apparently complete inhibition resulted.

In order to obviate this difficulty, two sets of experiments have been carried out. The first consisted in running a parallel series of tubes, one of which was shaken, the other not. The second group of experiments consisted in a parallel series of cultures, one in test-tubes, the other in small flasks so that the agglutinated bacteria would be brought into much more intimate contact with the medium as a whole, which was in a thin layer at the bottom of the flasks, than could possibly occur in test-tube cultures. The effect of shaking is well demonstrated in the experiment shown in Table VII.

TABLE VII.

Effect of Shaking on the Inhibition of Methemoglobin Formation by Antipneumococcus Serum.

2 hrs. at 37°C.		1 cc. of hemoglobin solution in each tube.	Incubation at 37°C.				
Culture 0.5 cc.	Serum 0.5 cc.			10 min.	30 min.	1 hr.	Shaken 10 min.
Pneumococcus Type I	Antipneumococcus Serum Type I	1 cc. of hemoglobin solution in each tube.	Unshaken.	—	—	—	++
“ “ I	Antipneumococcus Serum Type I		Shaken.	++	++	++	

Suitable control tubes showed that normal horse serum did not inhibit methemoglobin formation and that shaking in itself did not change oxyhemoglobin to methemoglobin. While complete inhibition of methemoglobin formation took place in the unshaken tube in the presence of homologous antipneumococcus serum, in the tube that was shaken so that the agglutinated clumps of bacteria were more or less broken up and diffused throughout the medium no inhibition occurred.

Further experiments illustrating this point were made with two strains of *Streptococcus viridans* which, like the pneumococcus, transforms oxyhemoglobin to methemoglobin. Strain 1 normally grew diffusely in serum-free bouillon, but like many streptococci grew in granular clumps at the bottom of the culture tube in the presence of serum. By growing this streptococcus in tubes containing anti-pneumococcus serum, it was possible to inhibit completely for 24 hours the formation of methemoglobin as shown in Table VIII, demonstrable methemoglobin being present only after gradual diffusion of the medium took place.

TABLE VIII.

Inhibition of Methemoglobin Formation by Streptococcus viridans by Antipneumococcus Serum.

Hemoglobin solution 1 cc. +			Methemoglobin formation.		Character of growth.
Culture 0.00001 cc.	Serum 1 cc.		After 24 hrs.	After 48 hrs.	
<i>Streptococcus viridans</i> 1	Antipneumococcus	Serum	—	+	Sediment.
“ “ 1	Type I Antipneumococcus	Serum	—	+	“
“ “ 1	Type II Antipneumococcus		±	++	“
	Normal horse serum.				and dif-
“ “ 1	Bouillon 1 cc.		++	++	fuse. Diffuse.

This inhibition cannot be attributed to any specific immunity principle in the serum and is more probably brought about by the growth of the streptococci at the bottom of the culture tube and their consequent inability to act on the medium as a whole.

Streptococcus viridans 2 normally grew as a sediment in bouillon and produced methemoglobin slowly as the growth gradually extended up the sides of the tube and diffusion of the medium took place. By shaking a culture of *Streptococcus viridans* 2 so that the organisms were in continual intimate contact with the whole medium it was found that the transformation of oxyhemoglobin to methemoglobin took place rapidly and was complete by the end of 6 hours.

at which time the unshaken control tubes failed to show any trace of methemoglobin (Table IX).

TABLE IX.

Effect of Shaking on Methemoglobin Formation by Streptococcus viridans.

Materials used.	Methemoglobin formation after incubation at 37°C	
	3 hrs.	6 hrs.
<i>Streptococcus viridans</i> 2, 0.5 cc. + hemoglobin solution 1 cc. + bouillon 1 cc. (shaken).....	±	++
<i>Streptococcus viridans</i> 2, 0.5 cc. + hemoglobin solution 1 cc. + bouillon 1 cc. (unshaken).....	—	—
Hemoglobin solution 1 cc. + bouillon 1.5 cc. (shaken).....	—	—
“ “ “ 1 “ + “ 1.5 “ (unshaken).....	—	—

It is evident from this experiment that the rapid formation of methemoglobin by this streptococcus depended upon its ability to grow in intimate contact with the whole medium.

Comparison of the inhibitory power of immune serum in parallel series of test-tube cultures and flask cultures confirmed the results of the shaking experiments. It was found that while marked retardation of metabolic function as measured by the change in the medium occurred in the test-tube in the presence of homologous immune serum, no inhibition occurred in the flask cultures in which the agglutinated bacteria were able to come into intimate contact with the medium as a whole. This is clearly shown in the experiments recorded in Table X.

From these experiments it is evident that antipneumococcus serum exerted no inhibitory action on the fermentation of litmus milk or on the splitting of protein by the pneumococcus when the organisms were grown in flask cultures, in striking contrast with the results in the test-tube cultures. Identical results were obtained with respect to the fermentation of inulin.

These two groups of experiments seem to throw considerable doubt on the theory that antipneumococcus serum possesses the property of inhibiting the metabolic activities of the pneumococcus by virtue of a specific antiblastic immunity principle. They rather indicate

TABLE X.

Relation of the Inhibition of the Metabolic Activities of the Pneumococcus by Antipneumococcus Serum to the Ability of the Pneumococcus to Act on the Medium as a Whole.

Fermentation of Litmus Milk.

Litmus milk 4 cc. +		Test-tube cultures.			Flask cultures.		
Culture 0.000001 cc.	Serum 1 cc.	After 24 hrs.	After 48 hrs.	After 72 hrs.	After 24 hrs.	After 48 hrs.	After 72 hrs.
Pneumococcus Type II	Antipneumococcus Type I Serum	++	++	++	++	++	++
" " II	Antipneumococcus Type II Serum	-	+	±	++	++	++
" " II	Normal horse serum.	++	++	++	++	++	++

Digestion of Protein.

Broth 8 cc. +		Increase in amino nitrogen per cc. after 48 hrs. at 37°C	
Culture 0.000002 cc.	Serum 2 cc.	Test-tube cultures.	Flask cultures.
		mg.	mg.
Pneumococcus Type I	Antipneumococcus Serum Type I	0.031	0.169
" " I	" " " II	0.103	0.120
" " I	Normal horse serum.	0.106	0.154

that the inhibition which occurs under certain experimental conditions is in reality due to agglutination of the pneumococci by the immune serum and their consequent inability to come into intimate contact with the medium as a whole.

Inhibition of the Metabolic Activities of Staphylococcus pyogenes aureus by Antistaphylococcus Serum.

Characteristic metabolic functions of *Staphylococcus pyogenes aureus* are the liquefaction of gelatin, the formation of a golden pigment, and the transformation of oxyhemoglobin to a magenta-colored unstable compound, which is probably reduced hemoglobin.

A limited series of experiments has been carried out to determine the ability of antistaphylococcus serum to inhibit or retard these metabolic activities of *Staphylococcus pyogenes aureus*. The staphylococcus used in the experiments was obtained by blood culture from a case of staphylococcus septicemia and an autologous immune serum was prepared by the immunization of a rabbit with this staphylococcus.

Experiment 9. Inhibition of the Liquefaction of Gelatin by Antistaphylococcus Serum.—The liquefaction of gelatin is a characteristic metabolic function of *Staphylococcus pyogenes aureus*. In a series of experiments to determine the inhibitory action exerted by antistaphylococcus serum on this activity of the staphylococcus it was found that complete inhibition occurred. One of these experiments is shown in Table XI. Incubation of the culture tubes was carried out at 20°C. so that no agglutination of the staphylococci would occur. It should be noted that in the tube containing antistaphylococcus serum there was no apparent inhibition of growth of the staphylococci as compared with that in the control tube. In other words, although the liquefaction of gelatin was completely inhibited, the immune serum exerted no apparent antagonistic action towards those metabolic activities of the staphylococcus which are essential to its growth and multiplication.

TABLE XI.

Inhibition of the Liquefaction of Gelatin by Antistaphylococcus Serum.

Gelatin 4 cc. +		Liquefaction of gelatin after incubation at 20°C.		
Culture 0.000001 cc.	Serum 1 cc.	3 days.	6 days.	9 days.
<i>Staphylococcus pyogenes aureus</i> .	Antistaphylococcus serum.	—	—	—
" " "	Normal rabbit serum.	++	++	++

Experiment 10. Inhibition of Pigment Formation by Antistaphylococcus Serum.—When staphylococci were grown on the surface of serum agar slants and in serum agar shake cultures, it was found that antistaphylococcus serum exerted no inhibitory effect on pigment formation by the organisms. It was felt that under these conditions the serum might not be in sufficient concentration in the immediate vicinity of the staphylococcus colonies to exhibit any demonstrable antiblastic action. In order to obviate this objection as far as possible, serum agar plates were prepared (serum 1 part to plain agar 4 parts) and a thin layer of serum was spread over the surface of the agar which was then streaked with a loop of

staphylococcus culture. The plates were sealed to prevent evaporation of the serum as far as possible and incubated at room temperature. Under these conditions the growing staphylococcus colonies were in intimate contact with the serum. It was found that antistaphylococcus serum definitely retarded the formation of pigment by *Staphylococcus pyogenes aureus* (Table XII), although it exerted no apparent inhibitory action on the rate of growth of the bacteria, as compared with the growth in the presence of normal serum.

TABLE XII.

Inhibition of Pigment Formation by Antistaphylococcus Serum.

Materials used.	Pigment formation.		
	After 24 hrs.	After 36 hrs.	After 48 hrs.
Antistaphylococcus serum agar + <i>Staphylococcus pyogenes aureus</i>	—	—	±
Normal serum agar + <i>Staphylococcus pyogenes aureus</i>	±	+	++

This inhibition of pigment formation by antistaphylococcus serum must be attributed to some definite antagonistic property of the serum toward this function of the staphylococcus. It should not be concluded, however, from this inhibitory phenomenon that the antagonistic action of the serum is definitely antienzymotic in nature, for it is not definitely known that pigment formation by *Staphylococcus pyogenes aureus* is caused by enzyme action. Certainly the formation of pigment is not essential to the life and growth of the staphylococcus, for it does not occur during the early stages of growth and appears only after the staphylococcus colonies are well developed and multiplication probably has in large measure ceased. Further experiments are necessary to establish the exact nature of this inhibitory action of antistaphylococcus serum.

Experiment 11. Inhibition of the Reduction of Oxyhemoglobin by Antistaphylococcus Serum.—To determine the inhibitory effect of antistaphylococcus serum on the reduction of oxyhemoglobin by *Staphylococcus pyogenes aureus*, two series of experiments were made, one with the use of a 5 per cent suspension of washed rabbit corpuscles, the other with a solution of hemoglobin prepared from rabbit corpuscles as in the pneumococcus experiments. A typical experiment is shown in Table XIII.

TABLE XIII.

Inhibition of the Reduction of Oxyhemoglobin by Antistaphylococcus Serum.

Suspension of rabbit corpuscles 1 cc. +		Reduction of oxyhemoglobin after incubation at 37°C.	
Culture 0.000001 cc.	Serum 0.5 cc.	24 hrs.	48 hrs.
<i>Staphylococcus pyogenes aureus.</i>	Antistaphylococcus serum.	++	++
" " "	Normal rabbit "	++	++
—	" " "	—	—

Hemoglobin solution 1 cc. +		Reduction of oxyhemoglobin after incubation at 37°C.	
Culture 0.000001 cc.	Serum 0.5 cc.	24 hrs.	48 hrs.
<i>Staphylococcus pyogenes aureus.</i>	Antistaphylococcus serum.	—	±
" " "	Normal rabbit "	++	++
—	" " "	—	—

It was found that the reduction of oxyhemoglobin was not inhibited by antistaphylococcus serum when rabbit corpuscles were used, but that inhibition occurred when a hemoglobin solution was substituted for the corpuscle suspension. This difference is readily explained on the basis of the results obtained in the pneumococcus experiments. In the tubes containing rabbit corpuscles the blood cells rapidly settled to the bottom of the tube where they were in intimate contact with the agglutinated staphylococci, and were therefore in a position to be acted upon by the organisms. In the tubes containing hemoglobin solution, however, the agglutinated staphylococci were not in intimate contact with the hemoglobin held in solution throughout the medium and consequently were unable to act upon it within the time limits of the experiment. When the agglutinated clumps of staphylococci were broken up and dispersed throughout the medium by shaking, the hemoglobin solution was rapidly reduced. From these experiments it is evident that antistaphylococcus serum possesses no specific antiblastic property which enables it to inhibit the reduction of oxyhemoglobin by *Staphylococcus pyogenes aureus*.

DISCUSSION.

The series of experiments reported in this paper were undertaken in an attempt to explain the mechanism by which immune serum is able to retard or inhibit the metabolic activities of bacteria. This property of immune serum has been attributed by previous investigators to an antienzymotic or antiblastic action of the serum which exerts itself at the point of contact of the bacterial cell with its environment. While it has been clearly demonstrated in confirmation of the work of other observers that under certain experimental conditions immune serum does exert an apparent inhibitory action on the nutritional and certain other metabolic processes of bacteria, the facts brought out as the experiments progressed have made it seem probable that the inhibitory action exerted by antipneumococcus serum is not due to a specific antienzymotic or antiblastic property of the serum, but rather to its agglutinating properties. It is well recognized that many bacteria grow readily in homologous immune serum, provided the serum does not possess definite bactericidal properties, a fact which in itself would tend to throw some doubt on the validity of the antiblastic theory as applicable to all immune sera, since metabolic activity is considered to be essential to bacterial nutrition and growth. Bacteria when cultivated in media containing immune serum grow in agglutinated clumps at the bottom of the culture tube. Under these conditions they do not come into intimate contact with the whole medium and are unable to bring about changes rapidly throughout the medium. Presumably the metabolic activities which bacteria carry on in the process of growth exert their influence on the medium only in the immediate vicinity of the agglutinated organisms during the early stages of growth, and only as gradual diffusion of the medium takes place are the bacteria able to act upon the whole medium in sufficient degree to bring about those changes which indicate that bacterial metabolism is taking place. That these considerations hold with respect to the influence exerted by antipneumococcus serum on the metabolic activities of the pneumococcus has been demonstrated in the experiments reported above, in which it has been shown that the metabolic activities of the pneumococcus do take place in the immediate vicinity of the agglutinated bacteria in spite of the

presence of immune serum; that the degree of apparent inhibition which antipneumococcus serum exerts upon the metabolic activities of the pneumococcus depends upon the extent of agglutination of the organisms; that antipneumococcus serum exhausted of its agglutinin content possesses no antiblastic properties; that antipneumococcus serum possesses the property of apparently inhibiting the metabolic activities of entirely unrelated bacteria provided those bacteria grow in sedimented clumps in the presence of the serum; and that when pneumococci are grown in the presence of antipneumococcus serum under conditions that enable them to come into intimate contact with the whole medium, no inhibition of metabolic activity takes place.

A limited number of experiments with antistaphylococcus serum has confirmed the results obtained with antipneumococcus serum in as far as the inhibitory effect of the serum on the reduction of oxyhemoglobin by the staphylococcus is concerned. On the other hand, antistaphylococcus serum has been found to inhibit or markedly retard the formation of pigment and the liquefaction of gelatin by *Staphylococcus pyogenes aureus*. Under the conditions of the experiments agglutination of the bacteria did not occur and therefore could not have been a factor in causing the inhibition. It is to be noted that neither pigment formation nor the liquefaction of gelatin is an essential process in the nutrition of the staphylococcus and that these phenomena take place in demonstrable amount only after the staphylococcus colonies have become well developed. It is felt that further experimental work is necessary to determine the exact mechanism of the inhibitory action of antistaphylococcus serum on these functions of the staphylococcus before the conclusion can be safely drawn that it is of an antienzymotic nature. The outstanding fact that staphylococci as well as pneumococci grow rapidly and abundantly in the homologous immune serum is sufficient in itself to indicate that the immune serum possesses no properties in demonstrable amount antagonistic to those bacterial metabolic activities essential to the growth and multiplication of the bacteria.

CONCLUSIONS.

Antipneumococcus serum under certain conditions apparently inhibits or retards the metabolic activities of the homologous pneumococcus.

Antipneumococcus serum exhausted of its agglutinin content possesses no inhibitory properties.

The degree of inhibitory action of antipneumococcus serum parallels its agglutinating power.

No evidence has been found to indicate that the inhibition of the metabolic activities of the pneumococcus by antipneumococcus serum is due to a specific antienzymotic property of the serum.

The evidence which has been obtained indicates that the apparent inhibition of metabolic activity that occurs under certain conditions is due to agglutination of the pneumococci by the antipneumococcus serum and their consequent inability to grow in intimate contact with the whole medium.

Antistaphylococcus serum inhibits the liquefaction of gelatin and the formation of pigment by *Staphylococcus pyogenes aureus*. Further experimentation is necessary to determine the mechanism of this inhibitory action.

Antistaphylococcus serum does not inhibit those metabolic activities of *Staphylococcus pyogenes aureus* essential to the growth and multiplication of the organisms

ACUTE LOBAR PNEUMONIA.

PREVENTION AND SERUM TREATMENT.

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INTRODUCTION.

The importance of acute lobar pneumonia and the need for means to prevent its ravages are now generally recognized. Among the infectious diseases pneumonia ranks next to tuberculosis as a cause of death, and in many of our large cities it kills more persons each year than does tuberculosis itself. In the public mind pneumonia is generally thought to affect mainly the very young and the old and decrepit. As a matter of fact, over half the cases occur between the ages of 20 and 50, during the period of greatest activity.

Although it occurs chiefly in endemic form, small and even large epidemics are not unknown. It was the most serious disease which threatened the success of construction of the Panama Canal, and its prevalence in certain regions where large numbers of susceptible workers are brought together, as in the mines of South Africa, renders it of great economic importance. Its importance as a cause of sickness and death among soldiers is not generally recognized, at least in this country, in spite of the fact that in our Civil War, aside from dysentery and typhoid, it caused far more deaths than any other disease. With improvements in knowledge of preventing and treating the two former diseases, pneumonia bids fair in the present war to lead all diseases as a cause of death. Judging from the experience in European armies, both in times of war and in times of peace, pneumonia is especially likely to attack raw recruits. The experience among the small number of troops on the Mexican Border, where pneumonia occurred in epidemic form, should be a warning of what is likely to happen in our national army when large numbers of susceptible men are brought together during the winter months.

Up to a few years ago the problem of preventing the spread of pneumonia or reducing its destructive effects seemed almost hopeless. The fact that bacteria apparently identical with those causing pneumonia are found in the mouths of many normal persons seemed to render any proposed measures for the prevention of infection well-

nigh useless. Attempts to cure the disease by the serum of immune animals had also given very inconclusive or negative results.

During recent years there has been in progress in the Hospital of The Rockefeller Institute a study of acute lobar pneumonia with the special object of improving methods of treatment. Realizing that such improvements are most likely to follow increase in knowledge of the pathogenesis of the disease and especially knowledge concerning the etiologic agent itself, much time has been given to the study of the pneumococcus, to the study of its distribution, which is so necessary for a knowledge of the epidemiology of the disease, and especially to a study of its immunity reactions, in order that methods for specific treatment might be obtained. As a result of this study, certain observations have been made and methods have been devised which have a direct practical bearing upon diagnosis and treatment. These observations have been published from time to time in papers appearing in *The Journal of Experimental Medicine* and elsewhere. At the present time of emergency and stress, however, it seems desirable that whatever is of practical value in this work should be made easily accessible, and, with the hope of meeting this need, this monograph has been prepared.

In the studies which have been carried on at the Hospital of The Rockefeller Institute, attention has been confined to pneumonia of the lobar type, due to *Diplococcus pneumoniae*, though certain of the results obtained are undoubtedly applicable to lobular infections of the lung and to other pneumococcus infections as well. It is our purpose here, however, to consider only the application of bacteriologic and immunologic facts to the former disease.

It is not intended to present a complete description of this disease, the clinical features of which are well known. A brief general review of the bacteriology of pneumococcus, however, is included; but no attempt has been made to present a complete review of the entire subject. Much work which is undoubtedly important has not been discussed, and the writers have aimed chiefly to present the results of their own work and that of their associates in The Rockefeller Institute, and to describe the technique that has been there developed in the etiologic diagnosis and specific treatment of this disease. It is hoped that this monograph may be of aid to the

worker in the clinical laboratory who assists in diagnosis, to the public health worker who is attempting preventive measures, to the manufacturer who prepares immune serum, and to the clinician who carries out specific treatment. To meet all these requirements in a small space has demanded that the material be very much condensed and that, as far as possible, theoretic discussions be omitted. On the other hand, the effort has been made to describe methods accurately and with considerable attention to detail, in order that they may be employed even by those without very extensive bacteriologic training. It has been thought best to err on the side of too minute description of details and, in many cases, technical descriptions of very simple procedures, that are undoubtedly quite familiar to trained laboratory workers, are included.

The problems concerning pneumonia which remain to be solved are numerous, and the difficulties of successfully applying serum treatment to all forms of pneumonia still seem almost unsurmountable. Nevertheless, we now possess considerable knowledge concerning the mode of spread of the disease, more knowledge indeed than we have of some other diseases that are thought preventable, and we have perfected methods for successfully treating at least a part of the cases. We believe that there exists at present not only the justification for, but also the duty of attempting to apply these facts on a larger scale, especially at this time when special danger threatens a large part of our most valuable population, and when it is so important that human life be conserved. The application of these facts, however, cannot be effectively made in a crude haphazard manner. Sufficient supplies of serum of standard potency must be available and public health laboratories, hospital laboratories, and army field hospital laboratories must be equipped to carry out the necessary procedures. In addition, there is needed a very close cooperation between the laboratory workers and those actually engaged in the care of patients, and the latter, as well as the laboratory workers, should have knowledge of the principles concerned and of the laboratory methods employed.

DEFINITION AND ETIOLOGY.

Acute lobar pneumonia is an acute infectious disease, the characteristic pathologic feature of which is a uniformly diffuse, exudative inflammation of entire portions of one or more lobes of the lung. It has long been a question of dispute, however, whether the definition of the disease shall be based on pathologic, etiologic, or clinical features. In our opinion, as far as prevention and cure of the disease are concerned, it is of the greatest importance that the chief stress should be laid on the etiologic agent. Lobar inflammation of the lung may undoubtedly be caused by a number of different bacteria. The vast majority of the lesions, however, are caused by varieties of *Diplococcus pneumoniae*. Among 529 cases diagnosed from the clinical and pathologic features as acute lobar pneumonia, the following were the etiologic agents concerned:

<i>Diplococcus pneumoniae</i>	454
Friedländer's bacillus	3
<i>Bacillus influenzae</i>	6
<i>Streptococcus pyogenes</i>	7
<i>Streptococcus mucosus</i>	1
<i>Staphylococcus aureus</i>	3
Cases of mixed infection with combinations of <i>Staphylococcus aureus</i> , Friedländer's bacillus, <i>B. influenzae</i> , <i>Streptococcus pyogenes</i> , and <i>Streptococcus viridans</i>	6
Undetermined (most of them occurring before accurate methods for determining the etiologic agent had been devised).....	49
<hr/> Total.....	<hr/> 529

It is not important to discuss here whether the pulmonary lesions due to *Diplococcus pneumoniae* are identical with those produced by other microorganisms. In typical cases, possibly in all, differences are present. At the present time, however, the differences in lesions cannot be detected with certainty during the life of the patient. The cases due to different varieties of bacteria are clinically indistinguishable, though the typical cases due to certain organisms

may present certain fairly characteristic features. With further study of large series of cases in which the etiologic agent in the individual case is carefully determined, it may be possible to devise methods for making a differential diagnosis on clinical grounds. At present we must rely on the isolation and identification of the organism concerned in each individual case. While we recognize, therefore, that lobar pneumonia may be due to a number of different organisms, in our present study we prefer to limit the discussion to that form due to *Diplococcus pneumoniae*. As will be shown later, all pneumococci are not identical, but may be differentiated into several different types. This fact is of fundamental importance in all that concerns prevention and treatment of this disease, and, therefore, we believe that from the clinical standpoint, as well as from the bacteriologic, the cases due to the different types of pneumococci, as well as those due to other varieties of bacteria, should be considered distinct clinical entities. Instead of considering lobar pneumonia one disease, we should consider it a group of diseases. At present we can distinguish no constant clinical or pathologic differences among the cases due to the different types of pneumococci, though certain observations suggest that a clinical differentiation, even of these cases, may not be entirely impossible. Such a separation of the cases of pneumonia is entirely analogous to what we have become accustomed to in the typhoid-paratyphoid group of infections. Clinically all the cases due to this entire group of organisms are very similar. Their differentiation, however, has become necessary for epidemiologic and therapeutic reasons. It is true that the different types of organisms in the enteric group possess slight cultural differences in addition to immunologic ones, while the different types of pneumococci differ from each other, as far as we can detect at present, almost entirely in their immunity reactions. These latter differences, however, from the standpoint of prophylaxis and treatment, are much more important than cultural distinctions.

DIPLOCOCCUS PNEUMONIÆ.

In 1881 Pasteur (21) described an organism, recovered from the saliva of a case of rabies, which produced a fatal septicemia in rabbits and which he designated *microbe septicémique du salive*. Almost synchronously, but quite independently, Sternberg (27) in this country reported the occurrence of a similar coccus in the saliva of healthy individuals. The microorganism described by these two investigators is now recognized as pneumococcus, or *Diplococcus pneumonia*. At the time these observations were made, however, the pneumococcus was not known to be associated with disease in man. It was not until the systematic investigations of Fraenkel (4) several years later (1884-85) on the etiology of pneumonia, and the report of Weichselbaum (34) on the occurrence of the pneumococcus in the blood, organs, and exudates of a large series of cases of this disease, that the causal relationship of this organism to lobar pneumonia was established.

The original discovery of pneumococcus in the saliva of healthy individuals, and the later investigations which proved this same organism to be associated with one of the most fatal of the acute infectious diseases in man, forecast the wide distribution which this organism is now known to possess. The fact that pneumococcus may vegetate as a harmless saprophyte in the mouth secretions of normal persons, and that this same organism may give rise to severe and fatal infections in man is also foretold in the early history of its discovery. Subsequent work by numerous investigators has abundantly confirmed this curious paradox, but the interpretation of its significance in the epidemiology and specific therapy of pneumonia has been made possible only by the recognition of the biologic relationships of pneumococci by means of immunity reactions.

Morphology and Cultural and Other Biologic Characteristics.

Morphology.—Pneumococcus is a Gram-positive coccus which in the exudates of infected animals and on artificial media is typically a lance-shaped diplococcus. Variations in the size and form of the cocci may be noticed, however, in different cultures and even in the individual organisms present in the same culture. Oval, round, and

elongated bacillary forms may occur. In young, actively growing broth cultures of pneumococcus, chain formation is common, while in the same culture, after 18 hours' incubation, the organisms are usually arranged in pairs with only an occasional short chain. In older cultures of pneumococcus, the organisms often appear Gram-negative, swollen, with degenerated and involuted forms, resulting from disintegration and death of the cells. Pneumococcus is readily stained by all the usual aniline dyes. It possesses no flagella, is non-motile, and does not form spores. Capsules are easily demonstrable in animal exudates and, by appropriate methods, may be found in cultures from media enriched with blood or serum. The capsular substance is most marked in recently isolated virulent strains, and is readily lost on prolonged cultivation. In *Pneumococcus mucosus*, Type III, the capsule is so pronounced as to be frequently demonstrable by Gram's stain, by which method it appears as a negatively staining halo about the Gram-positive cell body. The exact significance of the capsule of pneumococcus is not known. That it may serve as a protective mechanism of the organism and that it may in some way be related to virulence, is suggested by the fact that capsular development is always much more marked when the organism is grown in animal tissues in which presumptively there is some opposition to its development. Study of a large number of strains of pneumococci shows that there are average differences in capsular development between Types I, II, III, and IV (page 499). Although in some strains of Type IV pneumococci capsular formation is well marked, in others it is quite irregular and at times impossible to demonstrate, even under the most favorable conditions. Type I pneumococci usually show well formed capsules, and in Type II pneumococci the capsules are quite easily demonstrable and are generally somewhat larger and denser than in Type I. In Type III pneumococci capsules are always well developed, are very large in comparison with the size of the organisms, and may appear as voluminous envelopes about the cell bodies. In our experience the amount of capsular development seems to parallel the virulence of these organisms for man, as measured by the mortality occasioned by the different types in human infection. In addition, it seems to be true that the greater the amount of capsular development, the less the amount of passive protection afforded by immune serum.

Cultural Characteristics.—While growth may be obtained on the usual simple media, pneumococcus is, nevertheless, sensitive to slight variation in composition and reaction of the media. On media prepared from meat extract, growth is irregular and at best sparse and delicate. The pneumococcus colony on the surface of meat infusion agar, which has been enriched by the addition of a small amount of sterile defibrinated rabbit blood, presents certain characteristic features. The colony is small, moist, translucent, discrete, and round, with well defined edges, greenish in color, with a checker-like or ringed topography. The colony is finely granular, with a darker center, surrounded by lighter ringed areas. Pneumococcus on blood media produces a greenish zone of discoloration about the colonies.

In broth pneumococcus grows diffusely, producing a uniform turbidity without pellicle and with a slight flocculent sediment. The turbidity of broth cultures begins to clear up after 24 hours' incubation, corresponding to the period of beginning autolysis and the passing into solution of the disintegrating bacterial protein.

Gelatin is not liquefied; this medium, however, is not well adapted for the cultivation of pneumococcus, since no growth of this organism occurs at temperatures below 25°C.

In milk and inulin serum water pneumococcus produces acid and coagulation of the medium. Inulin fermentation by pneumococcus is of differential value in distinguishing this organism from streptococcus. In studying the fermentation of inulin by pneumococcus, various irregularities are encountered. Although fermentation is the rule, it is not uncommon to find strains that fail to ferment this carbohydrate, and, in addition, the same strain may ferment it at one time, and fail to ferment it at another. The failure to ferment inulin in many instances may be due to the fact that not all preparations of inulin are fermentable by pneumococcus, and not infrequently different lots of media, prepared from the same inulin, vary in their suitability for growth. Therefore, while inulin fermentation is of value in differential diagnosis of pneumococcus from other organisms, this reaction should be considered only as confirmatory evidence.

The addition of simple carbohydrates to culture media enhances the initial growth of pneumococcus. However, the amount of acid

formed by pneumococcus in sugar-containing media quickly reaches a point at which growth is inhibited and the organisms rapidly die in the products of their own metabolism. Fermentative reactions of certain bacteria on selective carbohydrate have, in some instances, furnished a basis for differential classification. A study of a large number of strains of pneumococcus of known types on a series of different carbohydrates has so far failed to show any fermentative reaction which would afford a biochemic differentiation corresponding to the immunologic classification of the four types of pneumococcus.

Temperature and Oxygen Requirements.—Pneumococcus is not sensitive in its oxygen requirement, and growth occurs equally well under aerobic and anaerobic conditions. The optimum temperature for cultivation is 37°C., the limits of temperature range being 25–41°C. The thermal death-point of pneumococcus as determined by Sternberg is 52°C. for 10 minutes. The viability of pneumococcus on artificial media is relatively short. It has long been a matter of comment that even on suitable medium of proper reaction pneumococcus sometimes fails to grow. This is especially common when an attempt is made to grow the pneumococcus in meat infusion broth. In making transfers from broth to broth it is usually necessary to increase the amount of inoculum in proportion to the amount of broth in which the organism is to be grown (R36).¹ The usual amount of culture that it is necessary to transfer in order to insure growth is about 0.1 cc. to 5 cc. of broth. It is usually easy to start growth in broth if the inoculation is made from infected blood. A loop of heart's blood of a mouse dead of pneumococcus septicemia often suffices to initiate growth in large amounts of broth, and always succeeds in amounts of 5 to 25 cc. of the medium. On the other hand, the transfer from broth to broth of pneumococcus recently isolated is more difficult and usually requires a considerably larger inoculation. As a strain becomes accustomed to growth in broth, reinoculations into this medium grow with more regularity, although always requiring the relatively large inoculum already referred to. Chesney (2) has shown that transfers of pneumococcus made during the period of maximum rate of growth grow readily and without the latent period of de-

¹ R before reference numbers indicates that the title appears among the papers by the Staff of the Hospital of The Rockefeller Institute (page 588).

velopment which is characteristic of older cultures. Kirkbride (6) has further shown that when transfers in broth are made at 8 hour intervals, pneumococcus maintains its animal virulence for long periods of time, whereas cultures made at 24 hour periods rapidly lose their virulence.

Viability.—The addition of defibrinated rabbit blood to media not only enhances the growth of pneumococcus, but prolongs the life of the culture. Cultures of pneumococcus in blood broth preserved in the ice box remain viable for several weeks. One of the best methods for preserving pneumococcus cultures is that devised by Heim (5), by storing the dried spleens of infected mice. At the time of autopsy the spleen of the infected mouse is removed and transferred to a small sterile glass tube. The infected organ is quickly dried in vacuum and the desiccated tissue kept sealed in the cold, unexposed to light. Pneumococcus thus preserved maintains its viability and virulence for months. The culture may be recovered by grinding up the desiccated spleen in a sterile mortar with a little broth and injecting the organ emulsion intraperitoneally into a mouse, from which the pneumococcus can in turn be recovered in culture. In selecting spleens for preservation, care should be taken to choose those from animals in which no mouse typhoid infection exists. Mouse typhoid is a common infection in mice, frequently responsible for the death of large numbers when it becomes widespread. Mice which are apparently healthy frequently carry the bacillus of mouse typhoid in the intestine, and when these animals are experimentally infected with pneumococcus, the typhoid organism becomes active and invades the blood, tissues, and body cavities. It is difficult to obtain pure cultures of pneumococcus from such mice because of the rapidity and vigor with which *Bacillus typhi murium* grows on artificial media, and because of its great virulence for these animals.

Bile Reaction.—Neufeld (13) first observed the fact that pneumococcus in the presence of bile undergoes solution. Bile solubility is now recognized as a distinctive biologic character of pneumococcus, and serves to differentiate it from closely allied organisms, such as streptococcus. The active substance responsible for the lytic action of bile on pneumococcus is cholic acid. In carrying out the test, whole bile or 10 per cent solution of sodium taurocholate or sodium

glycocholate in physiologic salt solution may be used. One-fifth to one-tenth volume of bile will cause solution of an actively growing broth culture of pneumococcus. Heat-killed pneumococci lose their bile solubility. The presence of serum in pneumococcus cultures inhibits or completely arrests the lytic action of bile. Neufeld reports the insolubility of certain avirulent strains of pneumococcus. This phenomenon of insolubility, however, must be comparatively rare, since among several hundred strains of pneumococcus isolated by us from lobar pneumonia, none has failed to be dissolved by bile. In the diagnosis, then, of virulent pneumococcus, considerable reliance may be placed on the bile reaction.

Virulence.—The virulence of pneumococcus is variable; it is attenuated by prolonged cultivation on artificial media, and increased by animal passage. The virulence of pneumococcus for one animal species does not necessarily imply similar invasiveness for the tissues of another. Pneumococcus recovered from lesions in man does not invariably possess increased virulence for susceptible animals. Pneumococci isolated from the blood of pneumonia patients, as a rule, are of such virulence that 0.000001 cc. of broth culture kills white mice in less than 36 hours. On the other hand, strains have been recovered from the same source which possess only moderate virulence, failing to kill white mice in much larger doses. The study of the virulence of pneumococcus isolated from the circulating blood during lobar pneumonia indicates that in general the intoxication is more severe and the prognosis graver, the greater the virulence of the infecting pneumococcus. Since, however, virulence can only be experimentally tested in a different species, and since the defensive mechanism of the host is variable, no direct measure of virulence in man is available. However, as will be shown later in the discussion of the biologic types of pneumococci, infection with pneumococci of the various types results in differences in mortality which are fairly constant.

In addition to pneumonia, pneumococcus may give rise to inflammation of the upper respiratory tract and the accessory sinuses of the nose. Infection of the middle ear and invasion of the meninges may also be due to pneumococcus. Primary peritonitis of pneumococcus origin may occur, especially in young children. Focal infections, as

empyema, peritonitis, pericarditis, endocarditis, arthritis, and lung abscess due to pneumococci, may occur as sequelæ of pneumonia.

Toxin Production.—Pneumococcus is a highly pathogenic micro-organism which is not known to secrete a soluble toxin, and whose harmful effects are supposed to be due either to the setting free of intracellular toxins, or to toxic split products formed by the disintegration of bacterial protein (R28), or possibly directly to the metabolic activities of the bacteria themselves (R29). When the bodies of pneumococci are dissolved either by the action of bile salts, autolysis, or by alternate freezing and thawing, the resulting bacterial solution is actively hemolytic for red blood corpuscles and is acutely toxic for animals (R18, R23). Intravenous injection of such solutions of pneumococcus produces death in rabbits and guinea pigs with acute symptoms resembling those of anaphylactic death. The hemolytic substance is thermolabile and its activity is lost by passage through a Berkefeld filter, and destroyed by proteolytic digestion with trypsin. Both the hemolytic and toxic properties of this substance are influenced by the same measures, and the potency of both is to some degree dependent upon the virulence of the pneumococcus from which the substance is derived. The lytic action of the hemotoxin, as well as its harmful effects in animals, may be inhibited by cholesterol and partially neutralized by immune serum. Neutralization by anti-pneumococcic serum, however, is incomplete and not strictly specific. Pneumococcus hemotoxin is not simply a product of autolysis, but undoubtedly exists preformed in the bacterial cell as a hemolytic endotoxin.

The production of methemoglobin by pneumococcus is another phenomenon the occurrence of which may in some way be concerned with the harmful effect of this organism in disease. The transformation of hemoglobin into methemoglobin by pneumococcus occurs only in the presence of free oxygen and the living bacterial cell. Culture filtrates and bacterial extracts of pneumococcus, when free from living organisms, do not induce the methemoglobin reaction.

Precipitable Substance (R6, R7).—Pneumococcus during the early stages of its growth forms a readily soluble substance which diffuses into the culture medium *in vitro*, and in human and animal infections is present in the circulating blood, whence it passes through the kid-

neys into the urine. This specific substance appears in the culture medium in considerable amounts as soon as the pneumococci start to grow. Its early presence is not attributable to the death and subsequent disintegration of the bacterial cell, but it is due to the extrusion into the medium of bacterial substance during the life processes of the organism.

One of the chief points of interest in the discovery of the soluble substance of pneumococcus is that it raises the question whether this substance is in any way responsible for the intoxication which attends pneumococcus infection. Studies to ascertain the answer to this question are being carried on at the present time, but have not yet progressed to the point at which a definite answer can be given. It may be said, however, that its toxicity is in no way comparable with that of diphtheria toxin. On the other hand, it possesses a degree of toxicity which, exhibited throughout the course of an infection, might account for the signs of intoxication in lobar pneumonia.

Differential Diagnosis.

Although a differential diagnosis is not always simple, pneumococcus possesses certain cardinal characteristics by virtue of which, in most instances, it may be readily distinguished from closely allied organisms, as streptococcus. Pneumococcus is bile-soluble, possesses a capsule, ferments inulin, is extremely pathogenic for mice, and on blood agar forms a small, moist, flat, ringed, checker-like colony with a greenish zone of methemoglobin about it. Moreover, as will be discussed later, the various types of pneumococci react specifically with their homologous immune sera. Streptococcus, on the other hand, is not bile-soluble, does not ferment inulin, is less virulent for mice; on blood media the colony is more opaque and raised, drier, and more coarsely granular, without the surface markings or ringed topography characteristic of pneumococcus, and is surrounded by a zone of either hemolysis or green pigmentation.

Considerable confusion still exists in bacteriologic literature regarding the identity of so called *Pneumococcus mucosus* and *Streptococcus mucosus*. *Pneumococcus mucosus*, or Type III pneumococcus, possesses the characteristics already described as typical of pneumococcus; it is bile-soluble, ferments inulin, possesses a voluminous cap-

sule, is extremely virulent for mice, and produces greenish discoloration of blood media. *Streptococcus mucosus*, on the other hand, while having a similar mucoid, heaped up, confluent colony, and possessing a capsule, is, however, not bile-soluble, does not split inulin, is not so pathogenic for mice, and has a greater tendency to produce hemolysis on blood agar. Finally, this organism, like other streptococci, does not react specifically with Antipneumococcic Serum Type III.

Biologic Classification.

With recent advances in bacteriology and the knowledge gained from attempts to apply the principles of bacterial immunity to human disease has come the recognition of the fact that, in addition to the grosser classification of bacteria, certain finer characteristics exist which serve to differentiate still further members of the same bacterial species. Through the work of Neufeld (16) and his associates, it was demonstrated that all races of pneumococci are not identical as far as their immunologic reactions are concerned. When the study of pneumonia at the Hospital of The Rockefeller Institute was undertaken, a large number of races of pneumococci from cases of pneumonia was isolated and the immune reactions of these were carefully studied (R33, R20, R35). Animals were immunized to each of these strains, and the immunity reactions of all of them to the various immune sera so obtained were studied. Not only was the agglutinability of the various races in each of the sera determined, but also the power of each of the sera to protect mice from infection with each of the strains. The results of these studies revealed the interesting fact that pneumococci fall into two general classes. The larger of these consists of pneumococci of Types I, II, and III, which comprise about 80 per cent of all strains encountered in disease, and which represent three apparently fixed types of highly parasitic organisms, each possessing common immunologic characters. Individual strains of Type I and Type II are characterized by the possession of immunity reactions common to all other members of the homologous group. A number of variants of Type II pneumococcus have been found (R4), a condition of considerable theoretic interest, the practical importance of which, however, is not sufficiently great to warrant detailed discussion here. Type III consists of *Pneumo-*

coccus mucosus, an organism distinguished from other types by morphologic and cultural, as well as immunologic differences, and related to other strains of the same type by common immunologic reactions (R38).

The smaller of these two main classes, which has been called Type IV, represents about 20 per cent of the strains isolated from cases of lobar pneumonia, and is the type most frequently encountered in the mouth secretions of normal individuals. Type IV pneumococci possess greater heterogeneity and consist for the most part of individual strains which are not interrelated. As originally described, this group was considered peculiar in that it seemed to comprise a heterogeneous series of independent varieties, none of which exhibited immunity reactions common to the other types or to each other. However, more recent study by Olmstead (19) of a large series of strains of *Pneumococcus* Type IV has shown that this group may contain smaller groups, the members of each one of which possess certain characteristic immunologic properties. These studies of Type IV pneumococcus are of interest and the results hold promise of practical application. At present, however, the work has not progressed to a point where practical use of it can be made, and hence it would be premature to discuss this group from the standpoint of specific therapy.

TABLE I.

Occurrence of Various Types of Pneumococcus in Lobar Pneumonia (454 Cases).

Pneumococcus type.	Incidence.	
		<i>per cent</i>
I	151	33.3
II	133	29.3
II (atypical)*	19	4.2
III	59	13.0
IV	92	20.3

*The incidence of atypical Type II pneumococci has been determined only during the last 2 years.

From Table I it is evident that *Pneumococcus* Type I is the most common cause of infection in man, giving rise to about one-third of the cases. Type II is next in frequency, and these two types together

give rise to over 60 per cent of cases of lobar pneumonia, at least in those localities in the United States in which studies of types of pneumococcus have been made. *Pneumococcus mucosus* (Type III) gives the lowest incidence in disease of the different types of pneumococcus. Type IV is responsible for about 20 per cent of cases, although the incidence of cases due to this organism seems to vary somewhat in different localities.

The accuracy with which these types may be differentiated and the constancy of their relative frequency in disease and health emphasize the importance of their recognition in clinical and epidemiologic studies. The exactness with which the large number of strains studied has conformed to type indicates the extraordinary uniformity and comparative fixity of the specific groups. These distinctive differences in immunologic properties of pneumococcus not only offer a reliable method for the more exact determination of the varieties of pneumococcus, but afford the only rational basis for the study of the epidemiology and immunotherapy of pneumococcal infections.

Culture Media and Staining Methods.

In the preparation of media suitable for growth of pneumococcus, attention should be paid to the following points:

1. All media should be prepared directly from infusion of fresh meat and not from beef extract.
2. Reaction of the media should be from 0.3 to 0.5 acid to phenolphthalein.
3. In the sterilization of the media, care should be taken to avoid excessive heating. Media should be sterilized by the Arnold method, 20 minutes on 3 successive days, and should not be autoclaved.

The following procedure has been found to give satisfactory results in the preparation of broth and agar for cultivation of pneumococcus:

Nutrient Broth and Agar.—1 pound of lean chopped beef is allowed to infuse in a liter of tap water over night on ice. The unfiltered meat infusion is boiled for 30 minutes, filtered through paper, and the loss by evaporation made up by the addition of water. 1 per cent peptone and 0.5 per cent sodium chloride are now added. 1.5 per cent agar may be added at this point if agar is desired. The mixture is allowed to boil for 2 minutes in the case of broth, or, if for agar, until the agar is dissolved. Titration to the neutral point with normal sodium hy-

dioxide is then carefully carried out. Media are then boiled for 6 or 7 minutes, made up to volume, and filtered clear, and sterilized in the Arnold sterilizer for 20 minutes on 3 successive days. The media should finally titer 0.3 to 0.5 acid to phenolphthalein.

Blood Agar.—For enrichment purposes, the addition of a small amount of sterile defibrinated rabbit blood to media prepared as above is recommended. The addition of three or four drops of sterile blood to 4 or 5 cc. of agar or bouillon serves as ample enrichment and provides most suitable medium for the growth and preservation of pneumococcus cultures. Blood may be obtained either from the ear vein of a rabbit or by direct heart puncture. Drawn directly into a small sterile container with glass beads, the defibrinated blood can be preserved for a week or more, and the method is, therefore, economical.

Serum Water Medium for Fermentation Tests.—For the determination of inulin fermentation by pneumococcus, the following medium devised by Hiss is used, a positive reaction being indicated by the production of acid and the coagulation of the serum protein. Clear beef serum is added to 2 or 3 volumes of distilled water. Heat the mixture for 15 minutes in an Arnold sterilizer at 100°C. to destroy ferments present in the serum. Add 5 per cent aqueous litmus solution to a concentration of 1 per cent or an amount sufficient to give a deep blue color. Add inulin to the serum water to a concentration of 1 per cent. The inulin may be best sterilized by autoclaving at 15 pounds' pressure for 15 minutes. Sterilize the inulin serum water by the fractional method, 100°C.

Preparation of Bile for Testing the Solubility of Pneumococcus.—Fresh ox bile, obtained directly from the slaughter house, is autoclaved for 20 minutes, at 15 pounds' pressure. The bile is filtered to remove the precipitate formed on heating and again autoclaved. The sterile bile is then ready for use. Heat does not destroy the lytic action of bile. While different samples of bile vary in their content of the salts of cholic acid and hence in their ability to dissolve pneumococcus, for routine purposes the bile reaction may be carried out by the addition of one-fifth to one-tenth volume of whole bile to plain broth culture of pneumococcus.

Stains for Pneumococcus. Gram's Stain (Sterling's Modification).—

Gentian violet	5 gm.
Alcohol, 95 per cent.....	10 cc.
Aniline oil.....	2 “
Distilled water.....	88 “

The aniline oil and alcohol are mixed by shaking, and the distilled water is added. The gentian violet is ground in a mortar and the aniline solution added while grinding. The solution is stable 3 to 4 months and stains rapidly.

Gram's Iodine Solution.—

Iodine.....	1 gm.
Potassium iodide.....	2 “
Dissolve in 20 cc. of distilled water. Add 300 cc. of distilled water.	

Safranin Counterstain.—10 per cent aqueous solution of a saturated alcoholic solution of safranin.

Hiss' Capsule Stain.—Preparations are best made by direct films from pneumococcus exudates. Dry in air and fix by heat. Stain for a few seconds with saturated alcoholic solution of fuchsin or gentian violet, 5 cc., in distilled water, 95 cc. Flood the slide with the dye and hold the preparation for a second over a free flame until it steams. Wash off the dye with 20 per cent aqueous copper sulfate solution. Blot; do not wash in water.

By this method the capsule appears as a faint blue halo around a dark purple cell body. Better results may frequently be obtained by omitting heat fixation and by washing off the dye with the copper sulfate solution as soon as it begins to steam. Water should not be applied at any stage of the procedure.

ETIOLOGIC DIAGNOSIS.

If we possessed no methods for specific treatment or for instituting specific preventive measures, the determination of the etiologic agent in each individual case of lobar pneumonia would not be of great importance. However, since it is now known that certain cases of pneumonia may be successfully treated with the serum of immune animals, provided that the organism used in preparing the serum is identical with that causing the infection, etiologic diagnosis becomes imperative. Practical and quite rapid methods have now been devised for the detection of the organism causing the disease, even for the differentiation of the specific types of pneumococci. This etiologic diagnosis is, however, not only of importance in relation to serum therapy, but has an important bearing on prognosis and is essential for acquiring knowledge concerning the epidemiology of this disease.

For the determination of the etiologic agent in cases due to other organisms than pneumococcus, the same general methods are applicable as in cases due to pneumococcus. In certain cases, as those due to Friedländer's bacillus, streptococcus, or staphylococcus, the microscopic examination of the sputum gives much information, and cultures of the sputum made on agar plates may show a great predominance of the organisms causing the disease. When the sputum from these cases is inoculated into a mouse, as in the method about to be described, these organisms outgrow the ordinary mouth saprophytes just as do pneumococci. Blood cultures from the patient, when positive, give, of course, clear evidence as to the nature of the infection.

In the cases due to pneumococcus it is important not only to determine that the infection is due to this bacterium, but it is also of prime importance to determine the specific type of pneumococcus causing the infection, since treatment with serum is only applicable in the cases due to Type I organisms.

The following is a description of the various procedures employed in the isolation of pneumococci and determination of the specific types:

Isolation of Pneumococci.

Organization of Laboratory.—In order that these methods may be of practical value, hospital and Board of Health laboratories should be so organized, if possible, that diagnoses can be made at any time, day or night. Arrangements for the rapid transmission of the specimen of sputum to the laboratory are of great importance, since the diagnosis must be made rapidly if the best results from serum therapy are to be obtained. After a moderate amount of experience by the laboratory worker, the method can be carried out with much rapidity, especially where a number of diagnoses are to be made at the same time; and where the work is properly organized, public health laboratories should not find that the introduction of this procedure into their routine adds a great burden. For the actual carrying out of the procedure, the comparatively simple equipment of any bacteriologic laboratory is sufficient. Facilities for the proper collection and microscopic examination of sputum, together with means for its inoculation into mice, and the simple requirements for mouse autopsy and bacterial cultivation are all the equipment necessary.

Collection of Sputum.—Care should be exercised in the collection of sputum to obtain a specimen from the deeper air passages, as free as possible from saliva. This can be done in practically all cases, even the most difficult, with a little persistence. The sputum is collected in a sterile Esmarch dish, or other suitable container, and should be sent at once to the laboratory for examination. When delay is unavoidable, the specimen should be kept on ice during the interval.

Microscopic Examination of Sputum.—Direct films of sputum are stained by Gram, by Ziehl-Neelsen, and by Hiss capsule stains. This serves to give an idea of the nature of the organisms present and an indication of the source of the sputum. Suitable lung specimens of sputum are relatively free, in most instances, from contaminating mouth organisms. It is frequently possible to identify Type III (*Pneumococcus mucosus*) organisms when they are present, as they possess very large, distinct capsules, staining by both Gram's and Hiss' methods.

Mouse Inoculation.—A small portion of the sputum, about the size of a bean, is selected and washed through three or four changes of

sterile salt solution in sterile Esmarch or Petri dishes to remove surface contaminations. When the sputum is too friable or when the specimen is relatively free from secondary organisms, this washing process may be omitted. In either event, the kernel of sputum selected is transferred to a sterile mortar, ground up, and emulsified with about 1 cc. of sterile bouillon or salt solution, added drop by drop, until a homogeneous emulsion is obtained that will readily pass through the needle of a small syringe. With a sterile syringe 0.5 to 1 cc. of this emulsion is inoculated intraperitoneally into a white mouse (Figs. 1, 2, and 3). The pneumococcus grows rapidly in the mouse peritoneum, while the majority of other organisms rapidly die off, with the exception of Friedländer's bacillus, *Bacillus influenzae*, and occasionally *Micrococcus catarrhalis*, staphylococcus, and streptococcus. Pneumococcal invasion of the blood stream also occurs early. *Bacillus influenzae*, if present, likewise invades the blood stream; other organisms, as a rule, do not. The time elapsing before there is sufficient growth of pneumococcus in the mouse peritoneum for the satisfactory determination of type varies with the individual case, depending upon the abundance of pneumococci in the specimen of sputum and the virulence and invasiveness of the strain present. It may be from 5 to 24 hours, averaging 6 to 8 hours with the parasitic fixed types I, II, and III. As soon as the injected mouse appears sick, a drop of peritoneal exudate is removed by means of peritoneal puncture with a sterile capillary pipette, spread on a slide, stained by Gram's method, and examined microscopically to determine whether there is an abundant growth of pneumococcus present. If there is an abundant growth of pneumococcus alone, the mouse is killed and the determination of type proceeded with. If the growth is only moderate, or if other organisms are present in any quantity, further time must be allowed until subsequent examination of the peritoneal exudate shows an abundant growth of pneumococcus. It should be emphasized that undue haste in killing the mouse is time lost in the end.

Mouse Autopsy (Fig. 4).—As soon as the mouse is killed or dies, the peritoneal cavity is opened with sterile precautions and cultures are made from the exudate in plain broth and on one-half of a blood agar plate. Films are made and stained for microscopic examination by Gram's stain and Hiss' capsule stain. The peritoneal exudate is then washed out by means of a sterile glass pipette with 4 to 5 cc. of sterile salt solution, the washings being placed in a centrifuge tube.

Cultures are then made from the heart's blood in plain broth and on the other half of the blood agar plate.

Determination of Types of Pneumococcus.

Agglutination Method.—When the pneumococcus is present in pure culture in the peritoneal exudate, the determination of type may satisfactorily be made by macroscopic agglutination tests, as follows: The peritoneal washings are centrifuged at low speed for a few minutes until the cells and fibrin contained in the exudate are thrown down. The supernatant bacterial suspension is transferred into a second centrifuge tube and centrifuged at high speed until the organisms are thrown out. The supernatant fluid is discarded and the bacterial sediment taken up in sufficient salt solution to make a moderately heavy suspension. The concentration of bacteria should be similar to that of a good 18 hour broth culture of pneumococcus. This suspension is used directly for macroscopic agglutination tests, being mixed with dilutions of immune serum in small test-tubes in equal quantities of 0.5 cc. each.

To obviate the difficulty that occasionally arises from the occurrence of Type IV strains that show cross agglutination in all three immune sera, the optimum dilutions of serum and the optimum incubation time that will surely identify all type strains and fail to give any cross agglutination reactions, have been determined on a large series of strains.² The results are shown in Table II.

TABLE II.

Determination of Pneumococcus Types by Agglutination.

Pneumococcus suspension 0.5 cc.	Serum I (1:20) 0.5 cc.	Serum II (undiluted) 0.5 cc.	Serum II (1:20) 0.5 cc.	Serum III (1:5) 0.5 cc.
Type I.....	++	—	—	—
“ II.....	—	++	++	—
Subgroups IIa, b, x.....	—	+	—	—
Type III.....	—	—	—	++
“ IV.....	—	—	—	—

Incubation for 1 hour at 37°C.

²The dilutions of immune serum indicated in both the agglutinin and precipitin tests refer only to the antipneumococcic serum in use at the Hospital of The Rockefeller Institute. As various lots of serum may differ in agglutinin and precipitin content, the concentration of serum required to assure specificity of reaction may vary somewhat from the figures given in Tables II and III.

From Table II it will be seen that a 1:20 dilution of Type I serum, making, with the addition of an equal amount of pneumococcus suspension, a final dilution of 1:40, a 1:20 dilution of Type II serum, making a final dilution of 1:40, and a 1:5 dilution of Type III serum, making a final dilution of 1:10, serve to agglutinate Types I, II, and III pneumococci respectively and fail to show any cross agglutination reaction with strains belonging to Type IV. It will further be seen that with 0.5 cc. of undiluted Type II serum, as well as with the 1:20 dilution, pneumococci belonging to the various subgroups of Type II may be identified and rapidly differentiated from Type II pneumococcus, in that they show partial to complete agglutination in undiluted Type II serum only, but not in the 1:20 dilution at the end of 1 hour's incubation at 37°C. For the determination of types of pneumococcus in the peritoneal washings, these serum dilutions give the most satisfactory and clear-cut results. Five small test-tubes are set up as follows:

Tube 1.	0.5 cc.	Serum	I (1:20)	+ 0.5 cc.	bacterial suspension.
" 2.	0.5 "	"	II (undiluted)	+ 0.5 "	" "
" 3.	0.5 "	"	II (1:20)	+ 0.5 "	" "
" 4.	0.5 "	"	III (1:5)	+ 0.5 "	" "
" 5.	0.1 "	sterile ox bile		+ 0.4 "	" "

Tube 5, containing bile plus bacterial suspension, is for the determination of the bile solubility of the strain and for the differentiation of pneumococcus from streptococcus. The tubes are incubated in the water bath for 1 hour at 37°C. Agglutination of Pneumococcus Types I, II, and III occurs promptly in these serum dilutions and is specific. Rapid clumping of the organisms is seen to take place and may be brought out clearly by gentle agitation of the tubes. For the identification of atypical Type II pneumococcus, incubation is necessary, these strains showing partial to atypical agglutination in undiluted Type II serum at the end of 1 hour's incubation. If no agglutination occurs in any of the serum tubes and the organism is bile-soluble, it is classified as Type IV pneumococcus.

Precipitin Method.—The determination of pneumococcus types by macroscopic agglutination tests with the peritoneal washings is interfered with when other organisms are present in considerable numbers, as occasionally happens, and consequently a delay of 18 hours

or more may occur before the type of pneumococcus present can be established. To obviate this, Blake (R9) has devised the following method: It has been shown that pneumococcus during the period of active growth in broth cultures produces a soluble substance which gives a specific precipitin reaction with homologous antipneumococcic serum. The method described by Blake is dependent upon this phenomenon. The peritoneal exudate is washed out with 4 to 5 cc. of sterile salt solution in the manner already described under the agglutination method, and the peritoneal washings containing cells, fibrin, and bacteria are immediately centrifuged at high speed until the supernatant fluid is water-clear. The supernatant fluid is then pipetted off with care not to disturb the sediment, and is mixed in quantities of 0.5 cc. each with equal amounts of antipneumococcic serum in a series of small test-tubes as follows:

Tube 1.	0.5 cc. Serum	I (1:10)	+ 0.5 cc. supernatant peritoneal washings.
" 2.	0.5 " "	II (undiluted)	+ 0.5 " " " "
" 3.	0.5 " "	II (1:10)	+ 0.5 " " " "
" 4.	0.5 " "	III (1:5)	+ 0.5 " " " "

An immediate specific precipitin reaction occurs in the tube containing homologous immune serum, the other tubes remaining clear (Table III). Incubation is usually not necessary. Two tubes of Type II serum are used for the purpose of distinguishing between Type II pneumococcus and members of its subgroups, the former giving a precipitin reaction in both tubes, the latter only in the undiluted Type II serum. A negative reaction in all serum tubes indicates pneumococcus belonging to Type IV. The precipitin method has been tested with a large number of strains and has been consistently positive and specific with *Pneumococcus* Types I, II, and III, and consistently negative with Type IV. The presence of other organisms, together with pneumococcus, in the peritoneal exudate does not interfere with the reaction, and other microorganisms than pneumococcus produce no substance that might give a false positive reaction.

The results with Subgroup II pneumococcus have not been so satisfactory. Reference to Table III will show that pneumococci belonging in these subgroups give a precipitin reaction with undiluted Type II serum, but not with the 1:10 dilution, thereby being distinguished

TABLE III.

Determination of Pneumococcus Types by the Precipitin Method.

Supernatant peritoneal washings 0.5 cc.	Serum I (1:10) 0.5 cc.	Serum II (undiluted) 0.5 cc.	Serum II (1:10) 0.5 cc.	Serum III (1:5) 0.5 cc.
Type I.....	++	—	—	—
“ II.....	—	++	++	—
Subgroups IIa, b, x.....	—	+	—	—
Type III.....	—	—	—	++
“ IV.....	—	—	—	—

from typical *Pneumococcus* Type II. A number of Subgroup II organisms, however, have been encountered in which the peritoneal washings have failed to give a precipitin reaction with undiluted Type II serum. In the identification of the fixed parasitic types of pneumococcus, this occasional difficulty is of little practical importance from the point of view of treatment, as there is at present no specific therapy for cases of pneumonia due to organisms of these types. For purposes of classification and statistics, these organisms can be readily identified subsequently when pure cultures have been obtained.

Confirmation of Types.—The determination of the type of pneumococcus by examination of the peritoneal washings should be confirmed by macroscopic agglutination tests with a pure bouillon culture of pneumococcus obtained from the heart's blood at the time of mouse autopsy. The technique is the same as that employed in the agglutination tests on the bacterial suspension obtained from the peritoneal washings. A test for bile solubility should also be made.

Determination of Type III Pneumococcus by Morphologic and Cultural Characteristics.

Of the four types of pneumococcus, the third type, *Pneumococcus mucosus*, is the only one which can be identified in most instances by morphologic and cultural differences. *Pneumococcus mucosus* is usually somewhat larger, rounder, and less lanceolate than other types of pneumococcus. It possesses a large distinct capsule which stains readily with Hiss' capsule stain and which usually retains the pink counterstain with Gram's method. The peritoneal exudate

of a mouse infected with this organism is usually quite mucoid and sticky, and the colonies on blood media are moist, mucoid, and confluent. Occasional strains, however, are encountered in which these characteristics are not so well marked and which are not so easily distinguishable by cultural or morphologic differences from other types. Furthermore, *Pneumococcus* Type II occasionally exhibits fairly well defined mucoid characteristics. For these reasons the identification of Type III pneumococcus by morphologic and cultural characteristics is not always absolute, and the diagnosis should be established by immunologic methods, when Type III serum is available.

Determination of Types of Pneumococcus in Blood Cultures, Spinal Fluids, Empyema Fluids, and by Lung Puncture.

Blood Culture.—From a positive bouillon blood culture, 10 cc. are removed by pipette and centrifuged at low speed to remove the blood cells. The supernatant fluid is pipetted off and the bacteria are thrown down by centrifuging at high speed. The supernatant fluid is discarded and the bacterial sediment resuspended in sterile salt solution. The type of pneumococcus is then determined by macroscopic agglutination tests following the technique described above.

Spinal Fluids and Empyema Fluids.—Cultures are made by the methods ordinarily employed in culturing these fluids, and the type of pneumococcus is determined by the agglutination method, with the same technique as that applied to blood cultures. If desired, in addition to culturing the fluids, a portion may be centrifuged at high speed to throw down the pneumococci present, and the bacterial residue may be taken up in 1 cc. of sterile salt solution and inoculated intraperitoneally into a mouse.

Lung Puncture.—This procedure should be resorted to only when it is impossible to obtain a suitable specimen of sputum or a positive blood culture. In our experience this procedure is necessary only on rare occasions. Cultures of the material obtained by puncture are made in bouillon, and the determination of type is made by the same technique as that employed in the case of blood cultures.

Determination of Types of Pneumococcus by Means of Specific Precipitin Reaction in the Urine.

The fact has been mentioned that a certain percentage of patients suffering from lobar pneumonia excrete in their urine at some stage of the disease a soluble substance of pneumococcus origin. This substance gives a specific precipitin reaction with antipneumococcic serum corresponding in type to the organism with which the individual is infected. A study of 111 cases of lobar pneumonia and closely related respiratory diseases has shown that in 65 per cent of the cases of pneumonia due to *Pneumococcus* Types I, II, and III, this substance is present in the urine and can be detected by means of the appropriate antipneumococcic serum. The reaction is specific for the type of pneumococcus with which the individual is infected, and in no instance of respiratory disease due to organisms other than pneumococcus has a positive reaction been obtained. The soluble substance may appear in the urine at as early a period as 12 hours after the initial chill, or it may appear for the first time at a later stage of the disease. It may continue to be excreted for many days after recovery has occurred. It is the rule to find this substance in the urine when pneumococcus septicemia exists. Examination of the urine for the presence of this substance has proven of some value for the rapid determination of the type of pneumococcus with which the individual is infected. Methods for the performance of the precipitin test are therefore given in detail. This test, however, should not supplant the usual bacteriologic methods for the differential diagnosis of pneumococcus types, but is useful in that in a certain percentage of instances an immediate diagnosis can be made from an examination of the urine.

Method Using Unconcentrated Urine.—A specimen of urine is obtained as soon as possible and cleared by centrifuging. The clear urine is mixed in quantities of 0.5 cc. each with equal amounts of antipneumococcic serum of the different types in a series of agglutination tubes (Table IV).

The strength of the reaction may vary from an almost imperceptible cloud to a heavy flocculent precipitate. The reaction may occur in some instances immediately on the mixing of urine and serum, or

TABLE IV.

Determination of Pneumococcus Types by Precipitin Reaction in Urine.

0.5 cc. urine from:		Serum I 0.5 cc.	Serum II 0.5 cc.	Serum III 0.5 cc.
Type I pneumonia.....		+	—	—
“ II “		—	+	—
“ III “		—	—	+
“ IV “		—	—	—

may require incubation in the water bath at 37°C. for 1 hour. Prolonged incubation, however, must be avoided, since bacterial growth under such conditions may obscure the test. In the precipitin reaction it is essential that all the reagents used, including the immune sera, should be water-clear. In case the reaction is negative or so faint as to be indecisive with the whole urine, the following method of concentrating the urine may be employed:

Concentration of Urine for Precipitin Test.—25 cc. or more of a 24 hour specimen of urine, with the addition of a few drops of acetic acid, are boiled down to a volume of about 5 cc., filtered through paper to remove any precipitate of albumin that may occur, and the filtrate is added to 8 to 10 volumes of 95 per cent alcohol. The precipitate which forms is collected by centrifuging, rapidly dried to remove the excess of alcohol, and the residue extracted with 2 to 3 cc. of salt solution, which redissolves the specific substance. Any undissolved material is removed by centrifuging and the clear salt solution extract is used for the precipitin test, as shown in Table IV.

LABORATORY AIDS IN PROGNOSIS.

Accurate prognosis in such an acute disease and one of such short duration as lobar pneumonia is very difficult. That the clinical picture may pass in the brief period of 24 hours from one that is apparently favorable to one of extreme urgency is a common observation. In the early stages of the disease it is almost impossible from the clinical picture alone to predict the outcome, and even in cases ending fatally, it is frequently difficult, even during the last days, to say what the result will be.

In the course of the studies on lobar pneumonia at the Hospital of The Rockefeller Institute some facts have been determined which are of value in estimating the probable severity of any case of pneumonia, and in predicting the result. Studies of the bacteriology of a large series of cases of lobar pneumonia have shown that the type of pneumococcus with which the individual is infected plays a part of considerable importance in the probable outcome of the disease. In spite of certain reports to the contrary, our experience has led us to believe that the presence of pneumococcus in the blood during lobar pneumonia indicates a severe infection and is of bad prognostic import. It has already been stated that during the course of lobar pneumonia there appears in the urine a soluble substance of pneumococcus origin. The quantity of this substance can be followed from day to day, and the amount excreted seems to be directly proportional to the severity of the infection. In occasional cases the blood picture is of value in forecasting the outcome of the disease.

Relation of Type of Pneumococcus to Mortality.

A study of a large number of strains of pneumococcus and the outcome of the disease in individuals infected with these strains has shown that the different types of pneumococcus vary in their virulence for man. In Table V are shown the percentage incidence of the different types of pneumococci in 100 cases of lobar pneumonia and the average mortality in those untreated with serum.

TABLE V.

Incidence of Various Types of Pneumococcus and Resulting Mortality.

Type of pneumococcus.	Incidence.	Mortality.
	<i>per cent</i>	<i>per cent</i>
I	33	25
II	31	32
III	12	45
IV	24	16

The statistics given in Table V approximate the facts as closely as they can be ascertained at the present time. Organisms of Type IV have in our experience been found to possess the lowest virulence of the different types for human beings. Type III pneumococcus, although it is the cause of the smallest percentage of cases, nevertheless gives rise to a high mortality, and the outlook for a patient infected with Type III pneumococcus is comparatively bad. Pneumococci of Types I and II are responsible for about 64 per cent of all cases of lobar pneumonia and are of relatively high virulence for man, the largest number of deaths in lobar pneumonia being due to these organisms. The mortality in Type I infection is 25 per cent, and in infections with Type II pneumococcus about 32 per cent. These two types together are responsible for approximately 62 per cent of all deaths from lobar pneumonia. It is evident, therefore, that the outcome of any case of lobar pneumonia is influenced by the type of organism with which the individual is infected, and knowledge of the type of pneumococcus causing the infection is therefore of value in prognosis. It must be borne in mind, however, that there is considerable variation in the severity of the individual cases due to the different types of pneumococcus.

Specific Precipitin Reaction in the Urine.

In the course of our studies it has been found that pneumococci of Types I, II, and III during the active period of growth form a soluble substance. Type III pneumococcus forms the largest amount of this substance, Type II somewhat less, and Type I the least. The elaboration of this substance by Type IV pneumococcus has not been

studied because of lack of specific sera for this group. This soluble substance is formed not only on artificial growth *in vitro* but also during natural infection in man. In patients suffering from lobar pneumonia this specific substance can sometimes be demonstrated in the blood. It is, however, frequently present in the urine in easily demonstrable amounts. The quantity of this substance present in the urine varies with the intensity of the infection and in many instances furnishes a basis for estimating the severity of the disease. In Table VI are presented the relative frequency of occurrence of the specific precipitin reaction in the urine during lobar pneumonia and the outcome of the disease in the cases studied.

TABLE VI.

Specific Precipitin Reaction in Urine in Lobar Pneumonia.

Type of pneumococcus.	Incidence.			Mortality.					
	No. of cases positive.	No. of cases negative.	Positive.	Cases showing positive reactions.			Cases showing negative reactions.		
				No.		Died.	No.		Died.
			<i>per cent</i>			<i>per cent</i>			<i>per cent</i>
I	20	15	57.1	20	2	10.0	15	0	0
II	20	8	71.4	20	10	50.0	8	0	0
III	12	5	70.5	12	7	58.3	5	1	20

The urine from 80 cases of pneumonia due to *Pneumococcus* Types I, II, and III was studied. In 28 of these the urine showed no reaction, and in 52, or 65 per cent, the test was positive. The mortality among the positive Type I cases was low, probably due to the fact that all these cases were treated with specific antipneumococcic serum. Of the 32 cases of Types II and III infection giving a positive urine reaction, 17 died, a mortality of 53.1 per cent. Of 13 cases due to *Pneumococcus* Types II and III having a negative reaction in the urine, only one died, a mortality of 7.7 per cent.

Most of the cases which fail to show the precipitable substance in the urine recover, whereas the mortality is high among cases in which its presence is demonstrable. The presence of specifically precipitable substance in the urine during lobar pneumonia therefore seems to indicate a severe infection. If the amount present in the

urine increases from day to day, the outcome is usually fatal unless this result is prevented by the administration of antipneumococcic serum.

Significance of Pneumococcus Septicemia.

The suggestion has been made that lobar pneumonia is primarily a blood infection, and that the local process in the lung is but part of a generalized condition. This view has arisen from the discovery that pneumococcus may at times appear in the circulating blood of persons suffering from lobar pneumonia. Reports in recent years have varied considerably as to the frequency with which it is possible to obtain pneumococcus from the blood. Some observers have obtained positive cultures in from 20 to 50 per cent of cases examined and, since these were the more severe cases, they think the presence of pneumococcus in the blood is of bad prognostic import. Others, claiming more suitable technique, have found pneumococcus in the blood during lobar pneumonia with great constancy and attach very little prognostic significance to its presence. In the cases of lobar pneumonia studied in the Hospital of The Rockefeller Institute, pneumococcus has not been found in the blood in all instances. In those cases in which a positive culture was obtained, the course of the infection has been invariably severe and in many instances the disease terminated fatally.

In Table VII are presented the results of blood cultures in a series of 448 cases of lobar pneumonia at the Hospital of The Rockefeller Institute.

TABLE VII.

Relation of Positive Blood Cultures to Mortality in Pneumonia.

Type of pneumococcus.	No. of cases examined.	Blood cultures.				Mortality.			
		Positive.		Negative.		Cases with positive blood culture.		Cases with negative blood culture.	
			per cent		per cent		per cent		per cent
I	145	50	34.5	95	65.5	13	26.0	3	3.1
II	148	49	33.1	99	66.9	36	73.4	9	9.0
III	55	16	29.0	39	71.0	16	100.0	11	28.2
IV	100	21	21.0	79	79.0	11	52.3	3	3.8
Total.....	448	136	30.3	312	69.7	76	55.8	26	8.3

Examination of Table VII shows that of 448 cases of lobar pneumonia, pneumococcus was obtained from the blood in 136 instances, or 30.3 per cent. In spite of the apparently low percentage of positive cultures in comparison with the statistics of some investigators, we feel that this percentage is representative of the number of positive blood cultures obtained by the ordinary hospital routine of taking one or two blood cultures in each case of pneumonia. The taking of repeated blood cultures during the course of the disease would probably raise this percentage. In 37 of these cases in which blood cultures were made at frequent intervals, positive findings were obtained in 50 per cent.

In the 136 cases with positive blood culture the mortality was 55.8 per cent, whereas in 312 cases with negative blood culture the mortality was but 8.3 per cent. These figures seem to show that the presence of pneumococcus in the blood during lobar pneumonia indicates a severe infection and is of bad prognostic significance.

The type of pneumococcus obtained from the blood has an important bearing on the outcome of the disease. In 50 cases in which Type I pneumococcus was obtained from the blood, the mortality was 26 per cent. The fact that this figure is so low is undoubtedly dependent upon the fact that a large number of these cases was treated with Antipneumococcic Serum Type I. Of 49 cases in which Type II pneumococcus was obtained from the blood, the mortality was 73.4 per cent. Of 16 cases in which Type III pneumococcus was recovered from the blood, the mortality was 100 per cent. Of 21 cases in which Type IV pneumococcus was found in the blood, the mortality was 52.3 per cent. These figures indicate that, with the exception of infections with Type I pneumococcus, in which case specific serum is efficacious, the more virulent the type of pneumococcus obtained from the blood, the less is the likelihood of recovery.

Our experience has been that the greater the number of colonies of pneumococcus that develop from 1 cc. of blood when plate cultures are made, the more likely the disease is to have a fatal termination. The following figures indicate the significance of increasing numbers of pneumococci per cc. of blood. The illustration is drawn from our experience with plate cultures made from the blood of individuals with Type II pneumococcus infections. Of 14 cases in which the

numbers of colonies were from 1 to 15 per cc. of blood, 6 died, a mortality of 43 per cent; of 2 cases with 15 to 30 colonies per cc., 2 died; of 4 cases with 30 to 75 colonies per cc., 4 died; of 16 cases with more than 75 per cc., 16 died. It is thus seen that all cases of Type II infection in which the number of colonies per cc. of blood was 15 or more were fatal. This figure, of course, is not to be considered as absolute, but serves to show that cases of Type II infection with considerable numbers of pneumococci per cc. of blood are almost invariably fatal. In some cases of lobar pneumonia, invasion of the blood may occur as late as the 5th or 6th day. A number of these cases are rapidly fatal and study of the blood has shown that the numbers of pneumococci per cc. of blood increased with extraordinary rapidity, the number of colonies in many instances running up into the thousands.

The study of these results leaves little doubt that invasion of the blood by pneumococci in any type of lobar pneumonia is a serious matter, that the seriousness of this invasion is proportional to the virulence of the type of organism, and that the greater the number of organisms present, the more likely is the disease to have a fatal outcome.

Technique of Blood Culture.—Under aseptic precautions 20 cc. of blood may be easily aspirated into a sterile Luer syringe from a convenient vein, usually near the bend of the elbow of the patient. The syringe may be sterilized by boiling for 20 minutes in a covered receptacle, or sterilization may be accomplished by dry heat or the autoclave, the syringe being enclosed in a large cotton-plugged test-tube. The latter method has the advantage of immediate availability when cultures are to be taken. The needles used are preferably nicked steel needles of about No. 18 gauge. The bevelled points should be sharpened each time before using, to insure ease in penetrating the vein and to reduce the discomfort of the patient to a minimum. With nervous individuals or in those cases where small veins or adipose tissue give promise of difficulty, the skin may be infiltrated with a little 1 per cent novocaine solution. Then, if any trouble is experienced in piercing the vein, the procedure will at least be painless.

When the blood is obtained 10 cc. are added to a flask of 150 cc. of

plain beef infusion broth (page 501) previously warmed to body temperature, and immediately incubated. The remainder of the blood is added in varying amounts to melted plain agar cooled down to about 45°C., and plates are made. Usually after 18 to 24 hours' incubation at 37°C., if the broth culture is positive, the supernatant fluid becomes diffusely turbid, and, on shaking, the mixture appears brownish red to chocolate in color. If very few organisms are present, several days may elapse before any change in the color of the cultures takes place. Consequently broth cultures should be incubated for 7 days and subcultures made on blood agar slants before they are considered sterile.

Colonies on the agar plates usually appear as small white dots, surrounded frequently by a greenish black area due to methemoglobin formation. Identification of the organisms obtained from the broth and agar cultures should be made by the methods described previously (page 511).

Leukocytosis.

It is well known that lobar pneumonia is usually accompanied by an increase in the total number of leukocytes in the blood, as well as by a relative and total increase in the percentage of polymorphonuclear cells. When such a leukocytosis is absent, it may mean a relatively mild infection or an overwhelming one. Chatard (1), in an analysis of 582 cases of pneumonia at the Johns Hopkins Hospital, found a mortality of 55 to 60 per cent in 39 cases having a leukocyte count under 10,000. With the rise in the number of leukocytes from 10,000 to 30,000 there was a progressive decrease in the mortality. Similar experience has been derived from observations of the leukocytes in 463 cases of lobar pneumonia in adults studied at the Hospital of The Rockefeller Institute. Table VIII shows the same striking increased mortality where the leukocytes were below 10,000 per cc., as found by Chatard, and a similar progressive fall in mortality with increasing leukocytosis.

While a single observation of white blood cells may furnish information of prognostic value, a study of the variation in the number and character of the leukocytes during the course of the disease has revealed many points of interest. It has been noted that

TABLE VIII.

The Relation of Leukocytes to Mortality in Lobar Pneumonia.

Leukocytes.	No. of cases.	Mortality.
		<i>per cent</i>
Under 10,000.....	29	65.5
10,000-20,000.....	143	23.7
20,000-30,000.....	177	18.0
30,000-40,000.....	76	14.4
40,000-50,000.....	29	24.1
Above 50,000.....	9	11.0
Total.....	463	

when consolidation of the lobe or lobes is taking place or during the period when a spread or infection of a new area of the lung is in progress, there may be a well marked decrease in the number of leukocytes. The following observations made on a patient having a Type I pneumococcus infection are illustrative:

Day of disease.	Leukocyte count.	Polymorpho-nuclears.	Lymphocytes.	Large mono-nuclears.	Temperature.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>°F.</i>
2	15,600	97	3		105
3	13,700	89	8	3	103.6
4	11,200	94	4	2	103.4
5	8,100	88	7	5	103.6

Lesion spreading from lower to upper lobe.

6	9,200	78	5	17	103
7	14,600	89	5	6	103.8
8	12,600	88	5	7	103.4
10	16,400	78	6	16	103
11	13,200	85	10	5	100
12	17,200	72	15	13	99.8
13	9,200	79	3	17	100.4
14	8,800	79	11	10	100.5

In this case a single observation made on the 5th and 6th days of the disease during the spread of the lesion from the lower to the upper lobe might have given one a false impression of the severity of the process.

A steady rise in the leukocyte count during the course of the disease is usually of favorable import. The following case illustrates this point:

Day of disease.	Leukocyte count.	Polymorphonuclears.	Lymphocytes.	Large mononuclears.	Transitionals.	Eosinophils.	Temperature.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>°F.</i>
4	9,400	93	4	3			103.6
5	9,400	96	4				
	13,600	87	4	7	2		103.6
6	14,400	88	5	5	2		103.2
	14,500	86	13		1		103.6
7	18,700	94	6				101.4
	20,000	86	4	5	5		100.2
8	14,400	84	6	5	4	1	99.2
	12,000	85	3	6	2	4	100
10	9,000	78	6		13	3	98.8
12	8,800	68	10	16	4	2	99.2

On the other hand, if an initial low leukocyte count shows little or no tendency to rise even with specific treatment, the prognosis may be grave. This is illustrated by the following case:

Day of disease.	Leukocyte count.	Temperature.
		<i>°F.</i>
3	8,000	102.5
4	7,200	102.8
	8,800	102.8
5	11,900	102.5
6	12,300	102
7	Died.	

With the fall in temperature by crisis or lysis, the number of leukocytes in the blood rapidly falls in the uncomplicated cases. A continuance of the high count, especially if accompanied by a continued high percentage of polymorphonuclear cells, suggests a complication with pus formation, most frequently empyema or otitis media. A continued leukocytosis with diminishing polymorphonuclear cells and corresponding rise in lymphocytic cells, with the presence of a few eosinophils, suggests delayed resolution, or serum sickness if the case has been treated with specific serum.

The appearance and staining qualities of the white blood cells often reflect the condition of the patient. In those mortally infected, in addition to an abnormally small number of leukocytes, there may be a predominance of degenerated polymorphonuclear cells. The nuclei of these cells appear fragmented, stain poorly, and the cytoplasm presents an appearance suggesting cloudy swelling.

On the other hand, a day or two before crisis, together with the increase in the total number of cells, there may appear many polymorphonuclear cells which may be termed immature forms. The nucleus is large and succulent in appearance and contains large amounts of chromatin material. The cytoplasm of the cell is, in addition, packed with well staining coarse granules. These minor changes that take place in the white blood cells in the presence of an infection, while of less practical value from the standpoint of prognosis than the information that may be derived from the determination of the type of infecting organism, the presence or absence of precipitable substance in the urine, or the occurrence of pneumococcus in the blood, are nevertheless of considerable importance.

ANTIPNEUMOCOCCIC SERUM.

It was demonstrated as early as 1891 that animals such as the rabbit can be rendered immune to lethal doses of pneumococci by the previous injection of sublethal doses of the organisms or by the injection of dead cultures. Furthermore, it was shown that the serum of these immune animals is able to protect other animals against a fatal infection, and even to cure them after infection has once occurred, provided that the injection of the serum is not too long delayed. It is not surprising that attempts were at once made to apply these fundamental facts to the treatment of lobar pneumonia in man. The serum of horses immunized to pneumococci has been widely employed in treatment (17, 20, 25, 26, 32, 33), but the results obtained have been inconclusive, and a few years ago the consensus of the best clinical opinion was that antipneumococcic serum was of little or no value.

It must be remembered that most of these efforts to treat pneumonia with immune serum were made before we had any knowledge concerning the immunologic differences between various races of pneumococci; they were all considered identical. Moreover, at the time these attempts were made to employ immune serum, there were no methods for accurate etiologic diagnosis in the individual cases, and the serum was administered to all cases indiscriminately. It is little wonder, therefore, that the results of treatment were unsatisfactory and inconclusive. Moreover, the immune sera were employed in small amounts and were injected subcutaneously.

We now know from experiments on animals that an antipneumococcic serum is only protective and curative provided it is employed to combat an infection due to the same type of organism as that used in its production. We also know that to cure after infection has once occurred, the serum must be employed in very large amounts, and preferably injected intravenously.

The discovery of these facts, together with the development of methods for making an etiologic diagnosis in every case, made it

advisable to undertake again the treatment of cases of pneumonia with the serum of horses immunized to the various specific types. These attempts have resulted in the demonstration that antipneumococcic serum, prepared by the injection of Type I pneumococcus, is highly effective in the treatment of cases of pneumonia due to the same type of organism. The serum of Type II is much less efficacious. Indeed, it has not yet been thoroughly demonstrated whether it has any practical effect on the outcome of the disease or not. Immune serum may be prepared by the injection of Type III pneumococci and this serum has slight agglutinating power and slight protective power for animals (30). This power, however, is so slight that the therapeutic application of this serum has not been considered promising or justifiable. From what has previously been stated it would be impossible to prepare a serum which would be effective against any considerable number of cases of infection due to Type IV pneumococci.

Production of Serum.

In spite of all that has been written concerning the theoretic principles involved in the preparation of antipneumococcic serum, and in spite of all the reports of the therapeutic application of various sera which have appeared, very little has been written concerning the actual method of preparation of these sera. In general, they have been prepared by the injection into horses at weekly intervals of gradually increasing doses, first of dead, then of living pneumococci.

Based on certain experimental studies which have been carried on in the Hospital of The Rockefeller Institute, this method of producing the serum has been modified, so that the time required has been materially lessened (R31).

What is attempted in the production of immune serum is to obtain, as rapidly as possible, a serum possessing the highest possible content of the known antibodies, agglutinins and bacteriotropic substances, and the highest possible protective action against pneumococcal infection, as tested in mice. Whether such a serum meets all the theoretic requirements is not at present our concern. Moreover, this serum should be specific; that is, effective against the type of pneumococcus causing the infection. Up to the present time antipneumo-

coccic serum has been demonstrated to be effective only in cases due to Type I pneumococcus, so in our opinion the production of anti-pneumococcic serum for routine practical use should, for the present, be confined to that prepared by the injection of this type of pneumococcus. At the present time the production of a polyvalent anti-pneumococcic serum is not practical or important, as it is in the case of the production of polyvalent meningococcic serum, for instance. The conditions as regards specificity among meningococci are quite different from those obtaining among pneumococci. The former organisms also differ among themselves in their immunity reactions. However, they all possess certain common characters, so that an immune serum against any one type has some action against all meningococci. With pneumococci, at least as regards the first three types, the immunity reactions are almost absolutely specific. A Type I serum is hardly more effective against infection due to Type II pneumococci than antidiphtheria serum would be. For these reasons we do not advise the routine manufacture of polyvalent antipneumococcic serum.

Concerning the size and spacing of doses, we have found from numerous experiments in rabbits that instead of the old method of weekly injections, much better results are obtained if the injections of dead organisms are made daily for a period of 7 days; then after another period of 7 days elapses, a similar series of injections is made. It has been proved that the doses should be small, as very large doses inhibit the immunity response. After several series of injections of dead culture, it may be necessary to employ the live bacteria. Here it is well to take advantage of the observations of Fornet and Müller, which have already been used by Flexner and his assistants in the manufacture of antimeningitic and antidyenteric serum. In this method the injections are made daily over a period of 3 days, determining the size of the dose largely by the reaction obtained. The following is a brief statement of the method of immunization as actually employed by us at present:

Technique of Immunizing Animals.—A sound, fairly heavy horse should be chosen and quarantined until a glanders test is performed. A specimen of blood obtained before any treatment is given is kept as a control. The immunization is carried on as follows: All in-

jections are made intravenously, employing for this purpose a Luer syringe. To avoid any accidental injury to the vein it is well to have the needle attached to the syringe with a small piece of rubber tubing. The strain of pneumococcus (living or dead) which is used for injection should be one highly virulent for mice (0.000001 cc. kills regularly) and it should have had very few passages either through animals or in artificial medium since removal from the human patient. The method for keeping these cultures is described above (page 495). In preparing the material for injection, both living and dead organisms, cultures are grown on beef peptone broth, reaction 0.3 to 0.5 per cent acid to phenolphthalein. Cultures about 12 to 15 hours old are preferable, as at this time maximum growth is present, with a minimum of autolysis. The cultures should contain about 200 to 300 million bacteria per cc. The culture is centrifuged until the supernatant fluid is clear. With the large centrifuge employed by us this requires about 20 minutes to an hour. The supernatant fluid is then pipetted off and the sediment is taken up in a small amount of sterile salt solution.

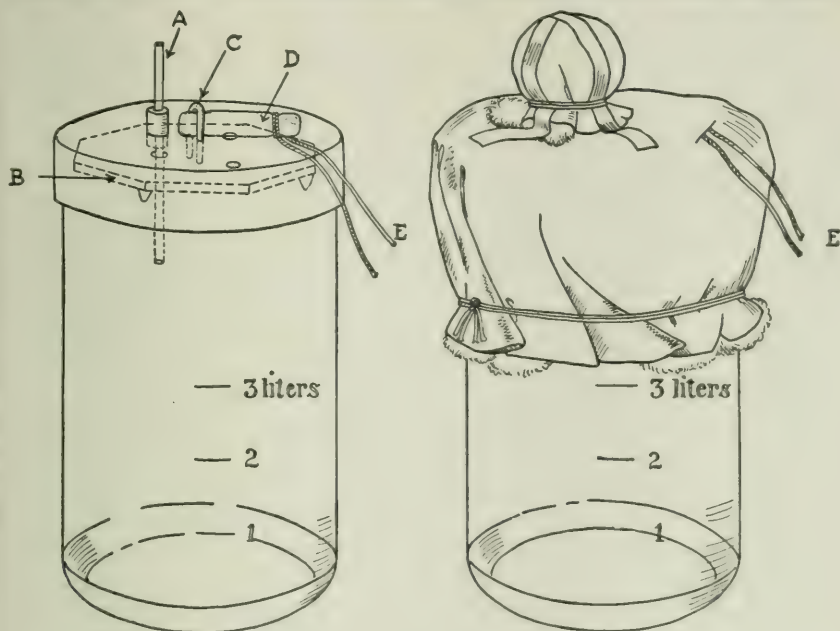
If living organisms are to be injected, the emulsion in salt solution is not made until just before injection, so that death and autolysis may not occur. If the organisms are to be injected dead, the emulsion is heated in a water bath at 56°C. for half an hour. For the daily injections a considerable amount of the vaccine is prepared as above and kept on ice. This vaccine may be employed for all the injections in one series of 6 to 7 days. We think, however, that a fresh emulsion should be prepared for each new series. Of course the emulsion of live bacteria should be freshly prepared for each injection. The dilution of the injected fluid should be such that the total volume is about 20 cc.

The following course of injections is now carried out: Every day for 6 days an injection of the emulsion containing bacteria from 50 cc. of bouillon culture is made. An interval of 7 days is allowed to elapse and then a second series of daily injections of the same sized doses is made. Again an interval is allowed to elapse and on the 6th day a specimen of blood is obtained for testing. Tests are made at once for agglutinating and protective power. This requires several days.

If the serum causes agglutination in a dilution of 1:200 and is of standard protective value, that is, 0.2 cc. protects a mouse against 0.1 cc. of virulent culture, bleeding could be carried out at once (10th to 12th day). As a matter of fact, we have never seen the titer of the serum after this amount of treatment to be so high. Consequently it has been our practice, 8 to 10 days after the last injection of the second series of dead cultures, to commence injections of living bacteria. Three injections are given on successive days. The temperature of the horse is taken every 2 hours for 8 to 10 hours following each of the injections of living bacteria. The first injection should contain the bacteria from 2.5 cc. of the original culture. If the temperature reaction following this injection is only moderate, not over 40.5°C., a second injection of the bacteria from 5 cc. growth is given on the following day. If the reaction from this is only moderate, the dose is again doubled on the following day, the bacteria from 10 cc. of bouillon being injected. It must be stated that as far as pneumococci are concerned it is difficult to regulate the dosage entirely by the febrile reaction obtained. If the reaction is very severe, of course the dose is made smaller than that mentioned. On the other hand, even though the reaction is very slight, we do not advise giving more than the amounts stated. 6 days after the last injection another specimen of blood is obtained for testing. If the serum is of the standard strength, bleeding can now be done. If, however, the potency is still too low, a second series of injections of living bacteria is made. These injections, unless there are reasons for modifying them, should consist of the bacteria from 20, 40, and 80 cc. of broth on the 3 days respectively. Again, the size of the doses may need to be somewhat modified, on account of the severity, or the lack, of febrile reaction. Most horses will require still further injections of living cultures. In any case, we think they should be made in series of three, given on successive days, with 7 day intervals between the series. The third series in most horses may consist of the bacteria from 100, 150 and 200 cc. of broth. From our present standpoint we believe that the size of an injection should never exceed the bacteria from 300 to 400 cc. of broth. Following the bleeding, it is well to allow the horse to remain quiet for 3 or 4 days. Then a series of three injections of living cultures, 50, 80, and 100 cc., is again given. After a week the serum is again

tested, and if of standard strength, bleeding may be done on the 10th day. If the serum is not sufficiently strong, a second series of injections of living bacteria is given, again keeping the dosage fairly small, never giving more than the bacteria from 300 to 400 cc., and always bleeding about 10 days after the last injection.

Technique of Obtaining Serum.—The bleeding of the horse usually takes place about 10 days after the last injection of antigen. It is possibly well to allow the horse to fast 12 to 18 hours before bleeding. The horse is then brushed thoroughly



TEXT-FIG. 1. Bleeding jars.

and its coat washed down with a dilute lysol solution. The floor, walls, and ceiling of the bleeding room are also washed down. The neck of the horse over the external jugular vein is shaved, washed with alcohol, painted with tincture of iodine, and anesthetized with a sterile 2 per cent solution of novocaine. The skin over the vein is incised and the vein is punctured with a cannula bearing a rubber tube, through which the blood is drawn into the receiving jar. This cannula is 8 cm. in length and 5 mm. in bore; the end of the cannula is bevelled for 1.5 cm., has a sharp point, and the bevel is turned against the blood stream. The bleeding jar (Text-fig. 1) is of glass, cylindric in shape, open at the top, 28 cm. in height, and 16 cm. in diameter, a slight modification of the type of jar

described by Kraus and Levaditi (8); we have found "Nonex" glass very satisfactory. The jar bears a loosely fitting cover of tinned copper having an eccentric and a concentric opening; the former carries an ordinary cork through which passes the glass, inflow tube (*A*), to which is connected the rubber tube from the cannula; the inflow tube reaches about 1 inch below the cover. Suspended from the cover, when it is in position on the jar, is a flat octangular weight (*B*) of tinned copper, with an aperture through which the inflow tube passes. This weight is 14.5 cm. in diameter and weighs about 700 gm. On the under surface of the weight are four projecting points or teeth, about $\frac{1}{2}$ inch in length, which serve to prevent it from slipping off the clot when dropped. The weight is suspended in the jar by a loop of wire (*C*) which passes through the concentric opening in the cover. By means of a small piece of wood or metal (*D*) passed through this loop, the weight is held in position. This piece of wood acts as a trigger which, when withdrawn, allows the weight to fall on the clot.

When the cover, weight, and inflow tube are in position on the jar, the whole is covered by a layer of paper which is tied around the jar. Through the paper the inflow tube is allowed to project. On top of this, the inflow tube still projecting, is placed a layer of cotton and another layer of paper, and these are tied securely; a cotton cap enclosed in paper is now secured over the inflow tube. A cord (*E*) attached to one end of the wooden support of the weight comes to the exterior through the paper and cotton covers. The jar so prepared is sterilized in the autoclave at 15 pounds' pressure for 20 minutes and afterwards dried at 100°C. by dry heat. It is advisable to place a few cc. of water in the jar before placing the latter in the autoclave.

Each jar is graduated and usually 3.5 liters of blood are drawn into each one. The cannula and tube are sterilized by boiling for 20 minutes. The cannula should have a sharp, cutting edge, which should be protected during sterilization by wrapping the instrument in cotton. When the cannula is withdrawn from the vein, usually no difficulty is experienced in stopping the blood flow, pressure and perhaps a stitch in the skin being sufficient in most cases.

6 to 8 hours after the bleeding, when the clot is firm, the trigger supporting the weight is released by pulling the attached cord (*E*); the weight then drops on the clot and materially aids in expressing the serum. The serum is usually drawn off the clot by syphonage about 40 hours after the bleeding, the short limb of the syphon being introduced into the jar through the central opening (now free) in the cover. The serum is syphoned into flasks of 6 liters' capacity. The long limb of the syphon passes into the flask through a doubly perforated rubber cork. Through the other perforation there passes a short cotton-plugged glass tube through which partial exhaustion of the flask starts the syphon action. Care should be taken in the syphonage not to carry over particles of clot or cellular débris. The serum is stored in these 6 liter flasks, sealed with rubber corks, and capped with sterile tin-foil. The syphon and corks are sterilized by boiling and the bottles (plugged with cotton and capped with paper) are sterilized in the

autoclave and afterwards dried at 100°C. by dry heat. Usually at a single bleeding 9 to 12 liters of blood are withdrawn and from this, employing the method described, we have usually obtained a yield of 50 to 55 per cent of serum.

Storage of Serum.—The serum is stored in the refrigerator in the 6 liter bottles for at least 2 months. During this period a fine precipitate gradually sediments. It is desirable that this sedimentation should be complete before the serum is dispensed for treatment in order to avoid the possibility of certain reactions on the part of the patient, due to the intravenous injection of suspended particles.

Dispensing Serum.—After the requisite period of storage, the serum is dispensed for treatment in sterile, rubber-corked bottles of 100 cc. capacity, capped with parchment which has been soaked in 1:20 phenol. This final bottling of the serum is accomplished by syphonage; this time, however, the syphon action is started by raising the air pressure in the storage flask through a cotton-plugged tube in the rubber stopper through which the syphon tube also passes. The syphon tube terminates in a specially designed filling device which, by means of a protecting bell or jacket, covers the neck of the dispensing bottle, thus avoiding unnecessary exposure of the serum.

All the handling of the serum is conducted in a room free from draughts and dust. After bottling, the serum is tested for agglutination and protective power and it is also subjected to a rigid test for sterility and for primary toxicity.

Tests for Sterility. Culture Tests.—The first, middle, and last of the dispensing bottles from each storage flask of serum are set aside for sterility tests. From each of these bottles, 0.5 cc. and 4 cc. of serum are each mixed with about 20 cc. of 1 per cent glucose broth in Smith fermentation tubes layered with paraffin; 2 cc. of serum are mixed with about 15 cc. of melted 1 per cent glucose agar at 43°C. in deep tubes, and, after cooling, layered with paraffin; 6 cc. of the serum are mixed with 150 cc. of ordinary nutrient broth; all are then incubated at 37.5°C., and observed for 14 days. If no growth or gas production is observed in any of the cultures, the particular lot of serum is considered sterile.

Animal Tests.—4 cc. of the serum are injected subcutaneously into each of two guinea pigs weighing 250 to 300 gm.; the guinea pigs are

observed for 2 weeks for evidence of toxicity or infection. 0.5 cc. of serum is also injected into each of two mice which are observed for 4 days. This test is for the purpose of making sure that the serum contains no living pneumococci from the last injection. However, in these tests we have never found pneumococci present.

Use of Antiseptics.—Employing the precautions we have mentioned, it has been rare for any lot or sample of serum to become contaminated. Where large amounts of serum are injected intravenously, as is necessary in this form of treatment, the ideal method is to employ serum to which no preservative has been added, and under the conditions in which we have employed the serum, we have shown that this is practical. The serum should, of course, be kept in the dark and in the cold. Under these conditions serum which we have kept several years has remained uncontaminated. If manufacturers feel that this method on a large scale is impractical, chloroform or tricresol may be added to the serum before bottling.

At present, most State Board of Health laboratories employ chloroform for the preservation of serum which is to be injected intravenously, and this is probably preferable to tricresol or other phenol preparations, though in the treatment of cholera large doses of immune serum, which have contained 0.5 per cent phenol, are said to have been employed (7) without injurious effects. Undoubtedly, however, where large amounts of serum are to be injected, the use of a volatile preservative, as chloroform, is preferable. The following is the method at present employed by the Department of Health of the City of New York: After the serum is drawn into the large flasks, an excess of chloroform is added, about 5 cc. to 1 liter. When the serum is drawn off for bottling, the excess of chloroform is left, together with the sediment, at the bottom of the flask.

Preservation of Potency.—We have examined samples of serum kept on ice and in the dark after a period of 2 years. No loss of strength could be detected by the methods used. If allowed to stand at room temperature and in the light, a fairly rapid loss of potency occurs. In the experience of Neufeld and Händel (14) also, antipneumococcic serum keeps many months unchanged. They also found that neither heating to 59°C. nor the addition of carbolic acid causes any loss in potency.

Concentration of Serum.—Experimental evidence and clinical experience have demonstrated that antipneumococcic serum must be administered in large doses if success is to be obtained in this method of therapy. Any method of concentrating the serum without decreasing its antibacterial potency, therefore, would be of great value in rendering its administration more convenient and practical. If concentrated serum were employed, moreover, the frequency of serum disease would be decreased, or at least the symptoms would be ameliorated.

Study of the distribution of the immune bodies occurring in antipneumococcic serum has shown that both the agglutinins and protective antibodies are associated or combined with the globulins, and not with the albumin of the serum (R2). Hence concentration by fractional precipitation of the globulin fraction with ammonium sulfate is possible. This procedure, however, is attended with certain disadvantages. Bacterial contamination of the serum necessarily occurs during the procedures attending the chemical manipulations. Although the bacteria may be removed by filtration through a Berkefeld filter and their growth inhibited by the addition of preservatives, the products of bacterial metabolism remain and may exhibit a toxic action when injected intravenously. It is doubtful, therefore, whether serum concentrated in this manner should be employed for intravenous use.

Gay and Chickering (R15, R16) have shown that the mixture of a solution of pneumococcus bodies and homologous antiserum results in the formation of a voluminous precipitate, which contains practically all the immune substances of the serum. The immune bodies contained in this precipitate can now be extracted in a dilute alkaline solution at 42°C. The resulting water-clear extract possesses the power to protect animals against pneumococcus infection and it also contains other demonstrable antibodies, such as agglutinins and precipitins. By this method a large proportion of the antibodies can be concentrated in a volume which is only one-fifth to one-tenth that of the original serum, and in which there is only one-sixtieth of the native protein. This biologic method of concentrating antipneumococcic serum, while yielding an end-product which is ideal in many ways, is nevertheless laborious in technique, and the preparations obtained are of very inconstant strength and are not stable.

While, therefore, concentration of antipneumococcic serum can be effected by either chemical or biologic methods, the disadvantages attending both of these processes are such that as yet neither has been found of practical utility.

Standardization of Serum.

In order that any serum may be used therapeutically, it is important that there should be some method of determining the strength or potency of the samples employed. Without this, no constant dosage can be obtained, and any estimate of the effectiveness of the serum is difficult or impossible. The method used for standardization of antitoxic sera, such as diphtheria and tetanus antitoxin, is almost ideal. Here it is possible to establish toxic and antitoxic units, and the law of multiple proportions holds good for these units within very wide limits; if 1 cc. of serum neutralizes 1,000 units of toxin, 0.001 cc. will neutralize 1 unit of toxin, or 100 cc. will neutralize 100,000 units, almost with the constancy of chemical acid-base neutralization.

An analogous method of standardization for antipneumococcic serum has been proposed, but there are great difficulties in the way of using such a method for this serum. No pneumococcus toxin has yet been demonstrated and the employment of an antitoxic unit is therefore impossible. Nevertheless, it might be possible to make use of the same principle of standardization, using bacteria themselves instead of their toxin, provided that a minimal lethal dose of bacteria could be established, and provided that the reaction between the minimal lethal doses and varying quantities of serum obeyed the law of multiple proportions. The attempt to employ such a method has been made by Eyre and Washbourn (3) and others. The establishment of a minimal lethal dose of pneumococci, however, is a matter of extreme difficulty. Pneumococci vary greatly in their virulence from time to time. With every passage through an animal or on artificial culture medium, an alteration occurs. This change may not appear very great, as estimated by our usual methods, but in relation to the great accuracy required in the establishment of a minimal lethal dose, the changes are enormous. It is only in organisms possessing the maximum degree of virulence that the virulence is at all stable. However, when cultures of high virulence are inoculated

into susceptible animals, as rabbits or mice, which are usually employed for these tests, it is found that very few bacteria, at times even a single organism, are sufficient to cause a fatal infection. With these bacteria the determination of the minimal lethal dose would be extremely difficult, if not impossible. Our studies indicate, however, that if such a unit could be established, the antiserum, when tested against it, would probably be found to obey the law of multiple proportions. Nevertheless, on account of the difficulty of accurately establishing a minimal lethal dose, this method does not at the present time seem practicable.

This failure to establish a unit for antipneumococcic serum, however, is not so serious as it might seem. The main practical object to be gained by the employment of this method of standardization would be that the dosage might be very accurately regulated, and that sera of weak power might be employed, provided that a sufficient amount were used. However, experience has shown that the maximum strength of any antipneumococcic serum that can be produced by present methods is limited. This limit is quite constant and differs for the different types of pneumococci employed. In order to obtain practical results in treatment, even with serum of this maximum strength, very large amounts must be used. Therefore, even if we had such a method of standardization as that above mentioned, it would not be practical to employ sera of low potency, nor would it be of great advantage to be able to estimate very accurately the exact potency of the dosage. Such a method of standardization of antipneumococcic serum therefore is, at the present time, neither possible nor necessary.

In the absence of an absolute standard, it might be possible to devise a test for determining the relative value of different sera, by making use of certain phenomena, as agglutination or opsonification. In these phenomena, if the amount of bacteria employed is not too great, the reaction is entirely proportional to the concentration of the serum in the mixture, not to its actual amount. At least, the only method we have at present for measuring the power of a serum to produce agglutination, consists in the determination of the maximum dilution at which the serum acts, and this is practically independent of the number of bacteria acted upon. There-

fore, if the effectiveness of a given serum depended upon its agglutinating power or were proportional to it, this property could be employed to fix the strength of any serum, and in such a method the great variable, the living bacteria, would be eliminated. Even in the absence of an absolute standard, we should then have a relative standard of efficiency. If bacteriotropic power were employed, however, the bacterial variable would not be eliminated, since it has been shown that different bacteria vary greatly in their susceptibility to the action of bacteriotropic sera, and this variation is to some degree dependent upon virulence. In any case, however, the hope of devising a method of standardization, employing either agglutination or bacteriotropic action, has been destroyed by the observation that, while there is frequently some gross quantitative relationship between the protective action of a serum and its agglutinating or bacteriotropic power, this relationship is inconstant. For instance, we have had sera with high protective power and little or no agglutinating power, and *vice versa*.

Although it is not possible to establish an anti-infection unit and although it is not possible to employ the properties of agglutination or opsonification in the ways we have mentioned for estimating the relative strength of sera, it is still possible to have a standard that will be of great practical value in the therapeutic employment of this serum. If a minimum potency for those sera that are to be employed therapeutically can be established, which minimum is not far below the maximum possible, we shall have gone far in standardizing the treatment of cases by serum, even though this method does not make possible fine distinctions in dosage or the use of sera of weak strength. It is, of course, necessary that serum of the strength determined upon can be produced without too great difficulty. Otherwise great waste would result and the whole method would become impractical. In view of what we have already stated in regard to agglutination and opsonification, these properties cannot be employed, but the potency of a given serum must be ascertained by direct determination of the protective power of the serum for animals. In order to establish by this method such a limit of potency as we have suggested above, there must be some constant relationship between the size of the infection dose and the amount of serum necessary to

protect; in other words, the serum must obey the law of multiple proportions.

Neufeld (14), who first studied this question, decided that antipneumococcic serum, as tested by protective power, obeyed this law only within given limits and under certain conditions. His experiments seemed to show that the results were regular until the serum had been diminished to a certain amount, depending on the weight of the animal employed. When an amount of serum less than this was employed, the relation of serum to culture no longer held constant; this smaller amount of serum would protect only against a very much less amount of culture than that calculated. From this he judged that to obtain effective action, even against minimal doses of culture, a given concentration of serum in the body (not actual amount) is required. This effective limit to the action of serum he called the *Schwellenwert*. His experiments were performed on mice and he did not mix the culture and serum before injection, but injected them separately in different parts of the body and usually allowed a period of time to elapse between the injection of the serum and that of the culture.

This matter was later studied in this hospital, but rats were employed and the given amounts of culture and serum were mixed in the syringe immediately before injection. It was found that the law of multiple proportions held valid as long as small amounts of culture and serum were employed; that even extraordinarily small amounts of serum would protect against proportionally small doses of culture. The relative amount of serum necessary to protect against larger doses of culture became greater and greater, however, as the size of the doses of culture was increased, until finally no amount of serum, however large, was able to protect.

The explanation of Neufeld's results lies in the fact that the serum was injected separately from the culture and, owing to the length of time required for diffusion of small amounts, multiplication of the bacteria had occurred and the degree of infection had increased enormously and the infection had become widespread before the serum became effective at the site of injection. Our experiments suggest that in the action of immune serum a body or tissue factor is essential, in addition to the substance contained in the serum. If the infection is not too great, this factor is adequate. If, however, the infection is enormous, this factor is no longer sufficient. Within given limits, however, any variations in this factor are not important and, moreover, within these limits the relation of serum to culture is fairly

constant. This fact is of great importance, as upon its validity depends the possibility of establishing a standard of potency.

From these experiments and those of Neufeld, it has been decided justifiable to fix a definite standard of strength to which sera used for therapeutic purposes should conform, determining this strength by direct estimations of the protective action of a definite fixed amount of the serum for mice against variable amounts of culture of high virulence. In such estimations as these it is obvious that while variations in virulence are important, slight variations make no great difference, as they would in determination of the minimal lethal dose, provided that the virulence is near maximum. The amount of serum to be employed in the tests also is not of great importance, provided that it is always the same and provided that it is selected within the limits where the law of multiple proportions holds valid.

Theoretically, it would be better to titrate several amounts of serum against varying amounts of culture, choosing these amounts of serum at various levels. Practically, however, this would render the method too complicated for ordinary use, and it has been found sufficient to use one amount of serum, provided that this amount is not too great or too small, and that the same amount is used for all tests.

Neufeld (17) used 0.2 cc. of serum for this purpose and we have found this satisfactory. Wadsworth and Kirkbride (30) have recommended using 0.1 cc. of serum without, however, giving any definite reasons for the change. Since much work has already been done using 0.2 cc., it seems best to continue this method.

We realize that the method of standardization which we advise is far from ideal. At the present time, however, it seems to be the only practical one, and, if applied with care and attention to details, it will at least prevent the use of sera of low potency, and, we believe, will make the therapeutic use of antipneumococcic serum both possible and effective.

Technique of Standardization Test.—In making a practical test of the protective power of the serum, white mice weighing 18 to 22 gm. are employed. The largest amount of an 18 hour broth culture of the homologous pneumococcus against which a constant quantity of the serum, 0.2 cc., will protect, is determined. Various dilutions of the broth culture are so prepared that each 0.5 cc. of the mixtures

contains a given quantity of the pneumococcus culture, from 0.2 cc. to 0.0000001 cc. The dilutions are made in peptone broth similar to that in which the bacteria are grown, and a separate pipette is used for making each dilution in order that an accurate distribution of the bacteria may be obtained, and to avoid carrying over any bacteria from the lower to the higher dilutions. The dilutions are made immediately before being used so that no growth or other change may occur in the mixtures. At the same time a small amount of the serum is diluted in broth, two parts of serum to three of broth, so that 0.2 cc. of serum is contained in each 0.5 cc. of the mixture. When injecting the animal, 0.5 cc. of the diluted serum is taken up into a 2 cc. Luer syringe and then 0.5 cc. of the diluted culture which is to be injected. The mixture of serum and culture is then injected at once intraperitoneally. The needle of the syringe is run under the skin a little above and parallel to Poupart's ligament and then obliquely through the abdominal wall. By this method injury to the liver, large blood vessels, or kidneys is avoided. In making a test of a given amount of serum, usually injections are made of 0.2, 0.1, 0.01, 0.001, and 0.0001 cc. of culture, each mixed with 0.2 cc. of the serum to be tested. It is best to inject two mice with each of these amounts. In making the series of injections one should commence with the mixtures containing the smallest amount of culture. It is then possible to use one syringe for all the injections. As a control of the virulence of the organisms used, another series of mice should be injected with these dilutions of culture, each with 0.2 cc. of the standard serum. Any marked variations in the virulence of the organism employed will then become evident, since by the protective power of the standard serum against the culture of organism employed, the virulence of the latter may be estimated. As a further control of the virulence of the organism, three mice are injected intraperitoneally, one receiving 0.0000001 cc., one 0.000001 cc., and one 0.00001 cc. of culture. The actual number of organisms contained in the culture employed is determined by making agar plates from each of these dilutions of culture and estimating the number of colonies appearing after 24 hours' incubation. With the same proportion of peptone in the broth and a standard acidity, it is surprising how nearly equal the numbers of bacteria contained in 1 cc. of each of the

various cultures are. Usually 0.0000001 cc. of culture gives rise to 10 to 30 colonies on the plate, which means that the number of bacteria contained in 1 cc. of the broth culture is from 100 to 300 million. It is our plan to mark the mice by staining a given part with carmine, and it is well to have a regular system for doing this and marking each animal before it is released from the hand. As the mice are injected, they are placed in glass jars containing sawdust and covered with pieces of wire netting. Care should be taken that they are not overcrowded (not more than three or four mice in a jar) and they should be kept in a room with an even temperature. They should be observed over a period of 5 days. With a virulent pneumococcus the controls, receiving culture alone, should die within 48 hours. These mice, as well as those unprotected by the serum, should have cultures made from the heart's blood in order to make sure that pneumococci are present and that some extraneous factor has not been the cause of death.

The best Type I sera that we have been able to produce have not protected regularly against doses higher than 0.2 to 0.5 cc. of culture. Most of them have not protected regularly against doses over 0.2 cc. We believe, therefore, that for therapeutic use only those sera should be employed which in doses of 0.2 cc. protect regularly against at least 0.1 cc. of a culture which is shown by the animal and standard serum tests to be of proper virulence. The virulence of the organism used in the protection test is usually such that 0.000001 cc. of an 18 hour broth culture will kill a mouse of 20 gm. weight within 48 hours. The following protocol illustrates the exact method of carrying out the test:

Typical Protocol of Standardization Test.

No. of mouse.	Marked.	Culture.	Serum.	Result.
Serum, Horse XX, Lot 4.				
		cc.	cc.	
1	Head.	0.2	0.2	D.* 48 hrs.
2	"	0.2	0.2	S. 5 days.
3	Back.	0.1	0.2	S. 5 "
4	"	0.1	0.2	S. 5 "
5	Right hind leg.	0.01	0.2	S. 5 "
6	" " "	0.01	0.2	S. 5 "
7	Left " "	0.001	0.2	S. 5 "
8	" " "	0.001	0.2	S. 5 "
9	Right fore "	0.0001	0.2	S. 5 "
10	" " "	0.0001	0.2	S. 5 "
Standard serum.				
11	Head.	0.5	0.2	D. 4 days.
12	Back.	0.4	0.2	D. 48 hrs.
13	Right hind leg.	0.3	0.2	S. 5 days.
14	Left " "	0.2	0.2	S. 5 "
15	Right fore "	0.1	0.2	S. 5 "
16	Left " "	0.01	0.2	S. 5 "
17	Head.	0.00001	—	D. 12 hrs.
18	Back.	0.000001	—	D. 24 "
19	Right hind leg.	0.0000001	—	D. 36 "

* D., died; S., survived.

In our experience sera produced by the injection of Type II organisms never regularly protect higher than in the ratio of 0.2 cc. of serum to 0.01 cc. of culture. The protective power of Type III serum is still less.

SERUM TREATMENT.

Indications for Serum Treatment.

It has been proposed by others that immediately after admission to a hospital or after coming under the care of a physician and before the type of infection is determined, every patient should receive a large dose of antipneumococcic serum of Type I or a polyvalent serum containing antibodies to Type I pneumococcus. The purpose of employing this method is to avoid the delay incident to the determination of the nature of the infection. Later, if the infection proves to be due to Type I pneumococcus, the treatment may be continued; otherwise it may be stopped. As we shall show later, the administration of serum is frequently followed by general constitutional reactions and, if this method were employed, many patients in whom the serum could be of no possible value would be subjected to these reactions. The time gained would not be great, since, in most cases, the type of infection can be determined within 6 to 18 hours, and during this time, if the patient is sensitive to horse serum, he can be desensitized. Pneumonia is the easiest of the acute infectious diseases to diagnose. If the importance of early diagnosis and of type determinations were generally recognized, serum treatment could be commenced very early in most patients, even though the etiologic diagnosis caused slight delay. We therefore believe that except in emergencies the administration of serum should only follow the determination of the nature of the infection.

As soon as the type of pneumococcus responsible for the infection has been determined, if it proves to be Type I, serum treatment should be undertaken at once in all cases, except in very young children who appear but little intoxicated by the infection and in whom the prognosis is good and the difficulty of treatment great. In the case of adults, first seen late in the disease, apparently mildly ill, and already showing signs of decreasing fever and intoxication, serum treatment may also be unnecessary. When cases are seen late

in the disease, still very ill, one should be extremely careful to eliminate complications as the cause of the symptoms. The value of the intravenous use of antipneumococcic serum in otitis media, empyema, pelvic or joint abscesses, pneumococcus pericarditis, or meningeal infections has not been demonstrated. Occasionally one is tempted not to use serum, or at least to wait a day or two, in cases which seem relatively mild at onset. This is believed to be most unwise, as frequently the pathologic process spreads insidiously, giving little evidence of intoxication for several days, when suddenly the picture changes, the pulse, temperature, and respiration rise, cyanosis deepens, and the end comes too soon to be influenced by the tardy use of the specific serum.

Occasionally one first sees a case at a time when consolidation of another lobe is taking place. At such a time the temperature may perhaps be lower and the heart rate and respirations slightly less rapid than previously. On the other hand, the general appearance of the patient does not seem reassuring; he may appear prostrated, and the extremities may be moist and clammy. In a few hours the temperature rises again, and the following day the signs of more extensive consolidation may be more apparent. These cases especially need immediate treatment when first seen if the further spread of the disease is to be prevented.

Administration of Serum.

Antipneumococcic serum can be administered most easily in a hospital where trained assistants and sterile materials are always available, but it may be given anywhere, in the home or in the field, in fact wherever water can be boiled and iodine and alcohol obtained.

Following the administration of foreign serum, especially the large amounts necessary in this form of therapy, the patient may exhibit certain symptoms which are entirely due to the foreign protein and have nothing to do with the content of the serum in antibodies. This reaction is identical with, or at least analogous to the so called anaphylactic reactions seen in animals. It is very important that, before serum is administered, every effort should be made to ascertain

whether patients are highly sensitive to horse serum, in order that such reactions may be guarded against. The detection of serum sensitiveness is not difficult. Every patient with pneumonia, immediately on admission to the hospital or after coming under the physician's care, should receive proper tests to determine this fact, since, if he proves to be infected with Type I pneumococcus, serum should be administered.

The patient is first questioned for a history of previous injections of immune sera for diphtheria, meningitis, or for tetanus infections. He is also questioned concerning previous symptoms suggesting asthma or hay fever, since persons having such symptoms are likely to be sensitive to various proteins, including those in serum. In any event, however, an intradermal skin test is performed.

Intradermal Skin Test.—The skin, preferably over the forearm, is carefully cleaned with soap and water and alcohol, taking care not to make the skin red or inflamed. If the skin is gently patted, instead of rubbed, with a gauze sponge, no irritation develops. With a small syringe, preferably of the type used for tuberculin injections, and a fine needle, about No. 28 gauge, 0.02 cc. of sterile diluted horse serum (normal or immune), diluted with salt solution 1:10, is injected *into* the skin, not subcutaneously, so that a small white wheal about 3 mm. in diameter showing the little depressions of the hair follicles is produced. An injection of a like amount of sterile salt solution is made as a control. It is advisable to make the serum and the control injections at one level on the forearm, about 3 to 4 cm. apart, in order that the test may not be obscured by the possible spreading of the injected material along the lymphatics.

The points of injection should be observed at intervals for 1 hour. Usually after a few minutes the wheal produced by the salt solution disappears, leaving no alteration in the skin except the small needle puncture. If the test is negative, that is, if no evidence is obtained of horse serum sensitiveness, the wheal produced by the serum injection fades away also almost as rapidly. On the other hand, if sensitiveness does exist, usually within 5 minutes a genuine urticarial wheal begins to develop, which may increase slowly in size up to that of a half dollar, the wheal in turn being surrounded by a larger area of erythema. This lesion usually reaches its maximum extent

within 1 hour and fades away rapidly, disappearing entirely within a few hours. A faint erythema may persist for a day or more. There are usually no subjective symptoms from this intradermal injection, except occasionally slight itching at the point of injection. Very rarely, in individuals extremely sensitive to horse serum, the injection of this small amount of serum, 0.002 cc., has been known to produce general symptoms, flushing of the face, increased rapidity of the heart rate, and respiratory discomfort.

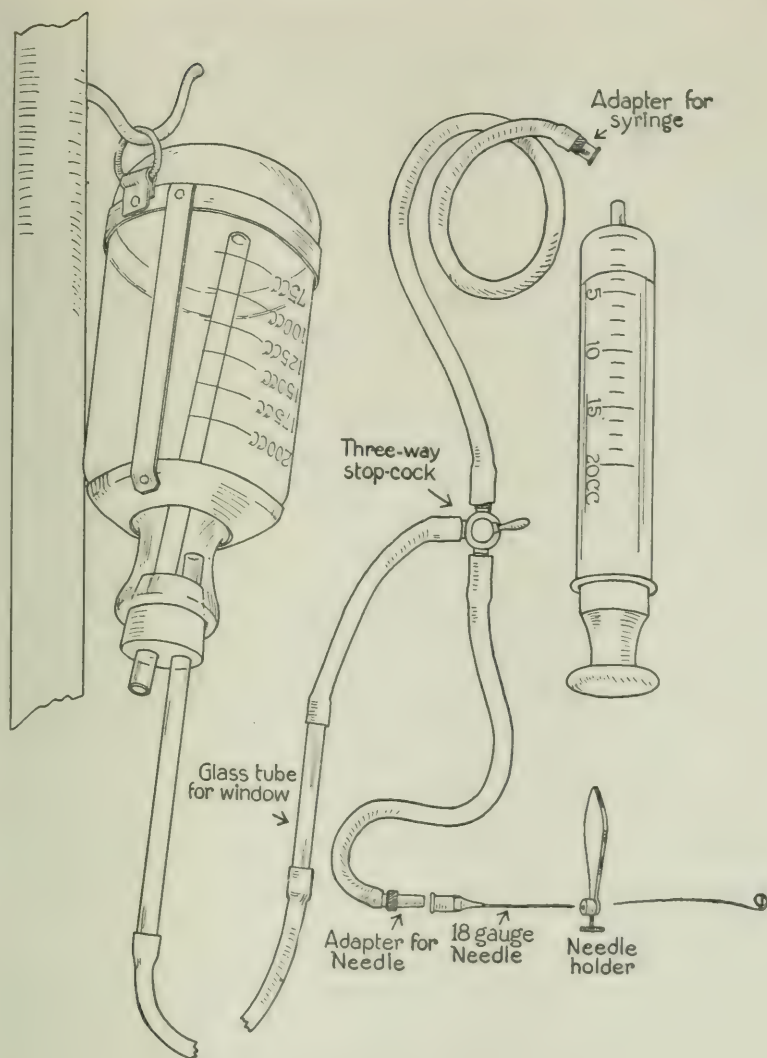
Desensitization.—It has been shown that even very susceptible and highly sensitized animals may be completely desensitized, so that they can tolerate large amounts of the serum to which they are sensitive, if repeated injections are made, beginning with an extremely minute dose and following this with doses of gradually increasing size. Even administering the serum with extreme slowness may be sufficient to prevent anaphylactic shock, or the administration of a very small dose, several hours before the large injection, may protect animals that are not highly sensitive. Therefore, for purposes of desensitization, it is advisable to inject 0.5 to 1 cc. of antipneumococcic serum or sterile normal horse serum subcutaneously into every pneumonia patient as soon as the skin test is completed, even though the intradermal skin test is negative, for occasionally one sees a patient in whom the skin test appears to be negative and yet in whom, on the injection of large amounts of serum, slight symptoms of serum sensitiveness appear. It is believed that the subcutaneous injection of a small dose of serum will desensitize a patient for from 6 to 8 hours to several days. However, this procedure should not be relied upon to the extent of giving the subsequent intravenous dose at a rapid rate. We know of at least one instance in which a desensitizing dose of 1 cc. of serum injected subcutaneously failed to prevent the development of a severe anaphylactic reaction in a patient who had received an intravenous dose of serum 10 days before. In our hands, however, the use of the preliminary intradermal and subcutaneous serum injections, we believe, has contributed much to our freedom from untoward symptoms during or after serum treatment.

If the intradermal skin test is positive, indicating that the patient is sensitive to horse serum and if the infection is due to Type I pneu-

nococcus, in which case the patient should receive serum treatment, a more thorough method for desensitization must be employed. This should consist in giving extremely small amounts of serum subcutaneously at $\frac{1}{2}$ hour intervals, doubling the size of the dose at each injection. One can safely begin with the injection of 0.025 cc. of serum. If no reaction follows the injection of 1 cc., subsequent doses may be given intravenously, also at $\frac{1}{2}$ hour intervals, beginning with 0.1 cc. and doubling the dose at each injection. If a general reaction occurs, or cyanosis, dyspnea, or increased rapidity of the heart rate supervenes, the injections should be suspended for 2 to 4 hours, depending upon the severity of the reaction, and then be resumed, starting with the same dose as that producing the reaction. After 25 cc. of serum have been given in these small doses, after a lapse of 4 hours, 50 cc. may be given, followed by the regular dose 6 to 8 hours later. The desensitization of a patient is a tedious and time-consuming procedure. Fortunately one rarely is called upon to employ this measure. It has been found necessary to desensitize in this way only two or three patients in a series of over 150 cases.

Technique of Treatment.—In preparing for the intravenous injection of serum it is best, though not necessary, to have a sterile field; that is, sterile towels, gloves, etc. It is, however, quite possible to inject serum aseptically with only a package of sterile gauze sponges and iodine and alcohol to disinfect the patient's skin about the point of injection. The apparatus for injecting the serum is shown in Text-fig. 2 and consists of the following:

1. Sterile container for mixture of antipneumococcic serum and sterile freshly distilled normal salt solution.
2. Rubber tubing, 4 feet long, and a three-way stop-cock.
3. Short piece of glass tubing for a window.
4. Rubber stopper with double holes through which are thrust two pieces of glass tubing. One tube is long enough to reach nearly to the bottom of the container, and through this air enters the container when the latter is inverted. The other shorter glass tube forms the outlet for the serum.
5. Two sharp needles and needle holder.
6. One 20 cc. Luer all glass syringe.
7. One 2 cc. Luer all glass syringe with hypodermic needles.
8. Thumb forceps.
9. Medicine glass.



TEXT-FIG. 2. Apparatus for injection of serum.

All this apparatus should be assembled and sterilized by boiling for 20 minutes in a covered dish. It is important to rinse out the tubing thoroughly after it has been used; otherwise with a subsequent sterilization any serum remaining in the tubing will be coagu-

lated and plug the apparatus. This material in the tubing may also be responsible for unexpected reactions on the part of the patient when it is accidentally injected along with the serum. To avoid this difficulty, the rubber tubing may be almost entirely replaced by lengths of glass tubing short enough to be contained in the sterilizing receptacle. These are connected by short pieces of rubber tubing 2 inches long, which are easily cleaned and can frequently be discarded for fresh ones. The serum should be warmed before injection to slightly above body temperature. It has been customary to dilute the antipneumococcic serum with equal parts of sterile freshly distilled (glass still) salt solution just before the injection is made. This makes the serum less viscous and facilitates its flow through the tubing. If salt solution properly prepared for intravenous use cannot be obtained, the serum may be used in its undiluted condition. The slow injection of the smaller amount of fluid during 25 to 30 minutes is, however, somewhat difficult.

When one is ready to make the injection, the serum is transferred to the container by means of a sterile pipette. In this way the transference of the small amount of sediment which is always present in the bottom of the bottle (if care has been taken to prevent shaking) can be avoided. If no pipette is at hand, the serum may be carefully poured from the bottle to the container, taking care that the sediment is not disturbed. This sediment may do no special harm, but its administration should be avoided if possible. When no container is available, the serum may be injected directly from the bottle, with a syringe with a long needle. The method described, however, is the one of choice.

Having filled the container, the cork is inserted and prevented from falling out by strips of adhesive tape. The bottle is inverted and attached to the side of the bed or to an irrigating stand. After allowing the serum to fill the tubing, the needle is introduced into a convenient vein, usually at the bend of the elbow, the overlying skin being previously cleansed with iodine and alcohol. If one has facilities for making blood cultures, it is wise to take about 10 to 15 cc. of blood for blood culture before beginning the serum injection. The serum may be allowed to run into the vein very slowly by gravity, but may be more easily and accurately controlled by aspirating it

into the syringe and then in turn injecting it into the vein (Fig. 5). In this way it is possible, and indeed advisable, that the steady and slow injection of the first 10 to 15 cc. of serum should occupy 10 to 15 minutes. During this period one watches carefully for any change in the patient's appearance and for increased rapidity of the pulse, respiratory difficulty, pallor, cyanosis, or urticaria. If these symptoms appear, it is well to stop the injection of serum for a few minutes, 15 to 20, to see if the symptoms increase in severity. Usually they rapidly disappear and the injection can be resumed. The needle can be easily kept free from clots by slowly injecting salt solution with the detached syringe. The serum can be kept at about body temperature when being injected slowly by placing the tubing between two hot water bags just before it reaches the patient.

Except in very rare instances, no symptoms arise during the injection of the first 10 or 15 cc. of serum, and the injection may then be completed more rapidly in 10 to 15 minutes. As soon as the needle is in the vein and is kept motionless, the patient experiences no discomfort; in fact, in most cases the patient dozes quietly during the treatment. With nervous individuals, the infiltration of a small area of the skin at the point of injection with 1 per cent cocaine or novocaine solution renders the whole procedure painless.

Dosage and Frequency of Treatments.

Practical experience has now shown that for antipneumococcic serum to be effective it must be given in large doses and intravenously, in order that a wide distribution of immune bodies throughout the body may occur rapidly. As previously stated, the exact mode of action of the serum is not yet certain; nevertheless to obtain satisfactory results, it is necessary to inject sufficient serum to produce in the patient's blood a concentration of antibodies such as that which occurs during natural recovery from the disease, for we believe that it is largely upon this factor that recovery depends (15, R32). If to obtain this result required merely the addition of a definite amount of the serum to the patient's blood, just as we might make a dilution of the serum in normal blood in the test-tube, there would be little difficulty. However, experiments have shown that (R

30) not only do the bacteria circulating in the blood fix antibodies and so render them ineffective, but in the infected patients the blood contains soluble substances which fix antibodies just as do the bacteria themselves. In the severely infected patients these soluble substances may be present in very large amounts, and it is only after these substances are all saturated that an effective concentration of immune bodies in the blood can be obtained. Moreover, it is not only necessary that the desired concentration of antibodies should be present immediately following the injection, but also that this concentration should be maintained. We have shown that as soon as this object is accomplished, unless the infection has lasted too long and has become too great, there occurs a fall of temperature and recovery from the disease.

The amount of serum necessary, therefore, will vary in each case. At present we have no means of deciding in the individual case what the primary dose should be. Neufeld, on the basis of the theory of *Schwellenwert*, decided that in the average man the dose should be about 75 cc. We also think the dose should be large and, from our experience and from the observations concerning neutralizing substances in the blood of infected patients, we have established more or less arbitrarily an initial dose of our standard serum of 90 to 100 cc. The frequency and size of the succeeding doses must be regulated largely by the effects obtained from those preceding. It was hoped that it might be possible to regulate the succeeding doses by determining the antibody content of the patient's blood at frequent intervals. This procedure is too complicated to be carried out regularly, even in hospitals that are best equipped for such work. In others this method is absolutely impossible. We must therefore be guided by the experience already gained from the careful study of a series of cases. We have concluded from these studies that it is extremely important that once having started specific treatment, it should be continued until a definite favorable result has been obtained and, in order to accomplish this, the serum should be given every 8 hours in doses of 90 to 100 cc. unless there are indications to the contrary. Our experience has indicated that the total amount of serum required may be diminished by active and persistent treatment at the start. The average total amount of serum required in our cases treated during the past winter has been about 250 cc.

In a considerable number of cases, within 20 minutes to an hour following the injection of serum there occurs an elevation of temperature, followed by a marked fall. This is described in the following pages as the so called thermal reaction. If the temperature continues low and the patient's condition is good, no further administration of serum is necessary. These patients, however, must be very carefully watched. The temperature should be taken every 2 hours and, if the temperature during the next 24 hours rises to 102°F. or over, a second dose of serum should at once be administered. If no fall of temperature occurs following the first dose, or if it does not fall to 102°F. within 8 hours, the second dose of serum should then be given. It is obvious that this rule should not be absolutely hard and fast, for the temperature is not the only guide to the patient's condition. However, it is better to err on the side of giving the serum too soon than of delaying too long.

If, following the second dose of serum, no reaction occurs, within 8 hours another similar dose should be administered, and this should be repeated as often as necessary. The patient should be observed day and night and the treatments should be given at night as well as by day whenever necessary. In the cases in which no reaction is obtained after several doses of serum, great care must be exercised to detect the presence of complications such as empyema or otitis media, to which the persistence of temperature may be due and on which the failure of the serum to act depends.

Serum Reactions.

The injection of serum may be followed by several types of reaction; first, the true anaphylactic reaction, second, the so called thermal reaction, and finally, usually at a later period, the symptom complex called serum sickness or serum disease.

Anaphylactic Reaction.—In individuals who are sensitive to horse serum, there may develop at once or within 15 or 20 minutes after the introduction of 40 to 100 cc. of serum, a more or less severe asthmatic attack with dyspnea and flushing of the face, followed by cyanosis, sweating, cough, and general anxiety, and an urticarial eruption. Methods have previously been described by which these

reactions can be avoided. According to Walker (31), however, reactions may occur, though very rarely, even in patients who have not reacted positively to horse serum and who have received a subcutaneous desensitizing dose. He has found that these individuals, though not sensitive to horse serum by the usual test, are very sensitive to the proteins in horse hair and horse dandruff. In these unusual cases small doses of horse serum produce no reaction, but doses of over 40 to 50 cc. of serum seem to induce a true anaphylactic attack. In these cases the first dose of serum usually produces complete desensitization and, following a second dose, even a very large one, no reaction of this type occurs. An anaphylactic reaction, unless extremely severe, is usually quickly relieved by the hypodermic injection of 0.6 cc. of epinephrin (1:1,000) solution or 0.5 mg. of atropine sulfate, or both.

It is the consensus of opinion among those who have had large experience in the administration of serum that severe anaphylactic reactions occur with great rarity, even when precautions have not been taken to avoid them. If all the precautions we have mentioned are taken, serious anaphylactic shock will probably never occur. The administration of antipneumococcic serum should never be neglected or omitted because of the remote possibility of the occurrence of these reactions.

Thermal Reaction.—Another type of reaction, that which follows the intravenous injection of various foreign proteins, the so called thermal reaction, appears usually from 20 minutes to 1 hour after the injection, and is characterized by chilly sensations or a general shaking chill, slight difficulty in breathing, and cyanosis. The temperature rises rapidly 1–3°F. and then falls, often to normal. During this fall there may be profuse perspiration. The patient usually rests quietly after a reaction and the general physical appearance seems improved. These reactions apparently have nothing to do with anaphylaxis or previous sensitization and may occur following the first dose of serum or following subsequent doses. Frequently the temperature remains normal, but in other cases after 6 to 24 hours the temperature again becomes elevated. It is this type of reaction which sometimes occurs following the administration of large doses of vaccines, and in such cases has been thought by some to have therapeutic value. We have

never felt that this reaction in itself is of benefit to the patient and believe that it should be avoided if possible. In certain instances administration of the serum too rapidly or the administration of cold serum may be responsible. Little treatment for this type of reaction need be given, reassurance, the application of heat to the extremities, and warm drinks being sufficient.

Serum Disease.—Following the administration of foreign serum, there frequently occurs a group of symptoms which together are called serum disease or serum sickness. The symptoms are fever, skin rashes, most frequently urticaria or erythema, edema of the skin, general glandular enlargement, and pains in the joints, and the symptoms may appear individually or in combination. The typical or most characteristic serum disease consists of all or most of the symptoms appearing together 7 to 14 days following the administration of serum, the attack lasting a few days to a week or more. The attacks may recur one or more times at intervals of a few days to a week or even longer. Such a typical and fairly well marked disease is not the rule, however. In most cases the course is extremely irregular. Certain of the symptoms, chiefly the skin rashes, may appear very early, within a day or a few days following the administration of serum. Later other symptoms may appear, singly or together. And these symptoms may reappear or persist irregularly for a month or more. Not all cases receiving serum show the symptoms. Mild symptoms of the disease appear in only about half of the treated cases, severe attacks only rarely or in not more than 10 per cent of the cases. The severity of the symptoms is not directly proportional to the amount of serum received, though undoubtedly the severe cases are more likely to occur in the patients that have received large doses of serum.

The fever in the mild cases may be very slight, hardly noticeable. In the severe cases it may be high, even reaching, exceptionally, 104° or 105°F. The cases with fever may cause much anxiety to the physician, since he is frequently in doubt as to whether the serum reaction is responsible for the fever or whether the fever is due to some complication. During the entire period of convalescence the physician should always be on his guard and, while he should realize that serum sickness may be accompanied by marked symptoms and

elevation of temperature, he should remember that it is unsafe to ascribe all unusual symptoms occurring during this period to serum disease. When fever occurs, frequent Roentgenographic examinations should be made to detect the presence of delayed resolution or the appearance of fluid in the chest. The occurrence of otitis media or localized infection should be looked for carefully. If the fever is due to serum disease, the patient does not usually appear very ill, as would be the case if the fever were associated with some focal complication with pus formation. The pulse is relatively less rapid, sweating is uncommon, and the appetite is good. The leukocyte count often helps in the differential diagnosis. While the total leukocyte count may be moderately elevated in serum sickness, up to 15,000 to 20,000, there is usually an increase in the relative number of lymphocytes, 30 to 40 per cent, and frequently the number of eosinophils is slightly increased, 3 to 7 per cent.

The skin rash may consist of only a few urticarial wheals appearing intermittently, or it may be widespread and consist of a diffuse erythema, even scarletiniiform, or it may be more irregular, suggesting measles. When the urticaria is extensive, pruritus may be very marked. This is usually the most distressing symptom. It may be relieved to some extent by allowing the patient to have a bottle of calamine lotion by the bedside which he applies to the itching areas as desired. Some such formula as the following may be used:

Carbolic acid.....	2 cc.
Calamine.....	4 gm.
Zinc oxide.....	8 "
Glycerol.....	12 cc.
Lime-water.....	16 "
Water up to.....	120 "

With this lotion the patient can usually be kept fairly comfortable during the day. At night the use of epinephrin hypodermatically, in doses of 0.6 to 1 cc., has been found useful. Following the injection the urticaria disappears like magic for a few minutes to a few hours, when it again returns. If one uses epinephrin at night, the patient will often fall asleep as soon as the urticaria disappears and will sleep several hours before being disturbed by the itching.

Occasionally there may be considerable edema present with the skin rash, or it may occur independently. It is most common about the face and neck and it may be so extensive as to close the eyes. This usually lasts only a few hours.

During the course of serum sickness there usually occurs slight enlargement of the superficial lymph glands. Sometimes only a few of the glands are enlarged; at other times the enlargement is general. The spleen may also become palpable, though this is not the rule. Occasionally the cervical glands beneath the sternomastoid muscle may become so enlarged and tender as to suggest a pyogenic infection, but this condition usually completely disappears in 2 or 3 days.

There is rarely nausea or vomiting. Very rarely acute abdominal pain may be complained of. There is no localized tenderness or rigidity, however, and this condition is usually transitory.

During the serum sickness, as von Pirquet and Schick (22) have noted, a disturbance of kidney function may be present. Longcope (11) found that about 10 per cent of his carefully studied cases showed albuminuria and hyaline and granular casts in the urine. There is little change in the coefficient of urea excretion or in the phthalein output. The excretion of water and chlorides is, however, profoundly affected, both being rapidly diminished with the onset of the sickness. This change in renal function is transitory and the return to normal is usually rapid.

Accompanying the other symptoms there may develop stiffness in the joints. The process seems to be extra-articular. There is rarely, if ever, any fluid in the joints, nor is the skin hot or reddened. There may be a slight amount of edema about the joints. All the joints may be attacked or only one or two. A joint frequently affected is the temporomandibular, and this causes considerable annoyance to the patient. Usually the joint symptoms pass away in a few days, but may last a week. The administration of aspirin or sodium salicylate and sodium bicarbonate usually gives relief from the discomfort. 0.3 to 0.6 gm. of the salicylate with 1 gm. of sodium bicarbonate may be given every 4 to 6 hours. Occasionally the patient complains of a headache which may persist for several days, but this is usually relieved by aspirin.

As previously stated, serum sickness may be very mild or it may be prolonged and troublesome, and the joint pains and urticaria may cause considerable distress. As far as we know, however, it is never serious, and leaves no permanent bad effects. Von Pirquet and Schick, whose monograph contains the best description of the disease, report no fatal cases, and we have never seen any serious effects, even in the worst cases. The fact that serum sickness may possibly occur should never deter the physician from giving serum. If life can be saved, the occasional occurrence of troublesome symptoms in a few patients is of little importance.

Supplementary Therapeutic Measures.

General Measures.—In addition to the specific therapy advised in the treatment of Pneumococcus Type I infections, the general hygienic and therapeutic management of the case must not be neglected. It is extremely important that the patient should be nursed, rather than nurse himself, from the onset of the infection. The prognosis in individuals who have pursued an ambulatory course during the first few days of the disease is distinctly worse. If transfer to a hospital is to be made, the patient should not be allowed to dress and walk to the ambulance, no matter how mild the infection may seem. Where impossible to move in the horizontal position, the transfer may be made semi-reclining in a chair. Even this exertion is often exhausting. The patient should not be allowed to help himself to food or drink, or to bedpans. The strict enforcement of these precautions plays an important part in the patient's recovery.

Diet.—The diet during the acute course of the disease is relatively unimportant. In general it should be fluid, easy to swallow and digest. Purées and soft boiled eggs, in addition to liquids, may be given if the patient desires them. In some cases whisky in small amounts may be beneficial. Water, 3,000 cc. or more a day, should be conscientiously given.

Treatment for Abdominal Distention.—The routine use of a daily morning enema of soap-suds aids in preventing this complication. Distention most frequently occurs in the severely intoxicated cases. It can frequently be controlled by proper diet, stupes, and medi-

cated enemata. If abdominal distention occurs, milk should be temporarily eliminated from the diet. Stupes may be conveniently given by applying compresses soaked in olive oil mixed with turpentine, 1 part of turpentine to 3 parts of oil, and covering with flannels wrung out in as hot water as the patient can tolerate, the heat being retained by a thick pad. The hot flannels should be changed frequently for a period of 20 minutes. A rectal tube may be inserted to aid in the passage of gas. This entire procedure is repeated at 20 minute intervals. To be effective, the application of stupes must be continued for a long period. Following their application, medicated enemata may be used. A very effective enema is one composed of ox gall 4 cc., turpentine 8 cc., asafetida 12 cc. This combination is diluted in 1 to 2 pints of soap-suds and is followed in 1 hour by a soap-suds enema. Usually this is effective in relieving the distention, at least for a short time. Some cases are temporarily benefited by the hypodermic use of pituitrin in doses of 0.5 cc.

Digitalis.—The experimental work of Porter and his coworkers (23) tends to show that the heart muscle is not vitally injured in pneumonia and that respiration usually fails before the circulation. Cohn (R17) has found that the pneumonic heart can be influenced by digitalis in quite the same way and by approximately the same dosage as the normal heart. Consequently it has been our custom to use some form of digitalis as a routine. On account of the convenience of administration and its accurate standardization, digipuratum has been used at the Hospital of The Rockefeller Institute, though any standardized form of digitalis may be used in corresponding doses. It is extremely important that the use of digitalis should be commenced early in the disease in all patients, in order that they may be partly digitalized before an immediate need arises. The intravenous use of digitalis or strophanthin is thereby avoided. The intravenous injection of these drugs may give rise to very serious symptoms, especially if the patient has previously received digitalis. Under this condition these drugs should never be injected into the vein. If patients are treated early, they are given 1 gm. of digipuratum at the rate of 0.5 gm. a day by mouth. If they are not seen until late in the disease and appear quite ill, 1 gm. is given on the first day. With this dosage there is usually evidence of the heart muscle being digitalized in about 24

hours. If the patient's condition does not indicate its further use, the drug is discontinued. If, however, occasion arises for digitalis later in the disease, it is again given at the rate of 0.5 gm. a day until the indication is satisfied, but it is not usually continued beyond 2 gm. The beneficial effects of the use of digitalis are best seen in cases of collapse, in which the pulse rate often rises to 150 to 180. This rise is usually due to flutter or fibrillation of the auricles. In these cases digitalis acts by blocking the irregular and frequent auricular impulses, so that often the ventricular rate falls below the initial rate and the symptoms of collapse disappear.

Optochin.—In 1911 Morgenroth and Levy (12) reported the discovery of optochin (ethylhydrocuprein), a derivative of quinine, which was found to have a specific bactericidal action on pneumococci both *in vitro* and *in vivo*. The experimental studies led to great hopes that this drug might be found to have specific curative effects in patients suffering from pneumonia. Extensive studies have been made on the drug in The Rockefeller Institute, both experimentally on animals by Moore (R43, R44, R45) and in the treatment of patients by Moore and Chesney (R12, R13). To our disappointment, however, in the treatment of 75 cases we have observed no definite beneficial effects. Because of this experience and owing to the danger of the drug producing injury to the eyes, the routine use of this drug in the treatment of acute lobar pneumonia cannot be recommended.

Results of Serum Treatment.

We shall here discuss only the effects of the administration of specific serum in the cases of lobar pneumonia due to infection with Type I pneumococcus.

General Effects.—We have already discussed the so called thermal reaction which may immediately follow the administration of serum. The fall of temperature associated with this reaction may be accompanied by amelioration of symptoms and the whole picture may present the features of a natural crisis. This occurs, however, in a small number of patients only. In other patients, following the fall of temperature and the relief of symptoms which almost always

accompanies it, there may occur a return of symptoms and rise of temperature, though, if serum is again promptly administered, this practically never reaches its previous height. In other cases there is little immediate effect of the serum on the temperature or pulse. In almost all cases, however, very soon after the administration of serum the patient's general condition improves. This is not only subjective, but is evident from the patient's better mental condition and increased interest in his surroundings. The cyanosis becomes less, and the pulse rate frequently falls. This phenomenon strongly suggests that, in addition to its other effects, antipneumococcic serum has a detoxifying effect. The exact nature of this effect however, can only be surmised. Beginning with this general improvement, unless the infection has been overwhelming, there occurs a gradual lowering of temperature and pulse rate, and amelioration of the other symptoms, the patient usually returning to a normal condition within a few days. What almost certainly happens following the administration of the serum is that the progress of the disease is stopped. How rapidly complete recovery may occur depends on numerous factors. That the progress of the disease is stopped if sufficient serum is given is made evident by the following facts, in addition to those just mentioned: First, in practically all cases, following the administration of the serum, there has been no extension of the local lesion. In certain cases in which the serum has been administered early, sometimes only a very small portion of a lobe has become consolidated. Second, invasion of the blood by pneumococci has been prevented (page 517). Slight invasion of the blood by bacteria probably always occurs at some time during the disease, at least in all but the very mild cases. In the more severe cases a true septicemia is usually present, and this always occurs in the cases ending fatally. In our opinion the fatal outcome is usually dependent upon this general infection. Now, in almost every case in which we have administered serum and in which the blood culture was positive before the administration of the serum, one dose of serum, or at the most two, has resulted in the blood becoming sterile. This fact seems to us of great importance in judging the efficacy of the serum. Third, following the administration of the serum, there appear in the patient's blood the immune

bodies which normally appear only at or about the time of crisis. It is possible, of course, that these immune bodies are only those contained in the serum administered. Whether the patient himself is stimulated or permitted to produce immune bodies more rapidly or in greater numbers than would otherwise be the case is not known.

We have previously stated (page 515) that the presence of precipitable substance in the urine is a bad prognostic sign. In several cases where the reaction was positive at the time the administration of serum was commenced, following the administration of serum the reaction became negative.

Effect on Resolution and the Development of Complications.—The administration of serum apparently causes no change in the rate of resolution of the lung tissue already involved. This process is apparently independent of immunity reactions, and consolidation of the lung may persist even after the temperature is normal and the patient is, from a superficial examination, well. There is no evidence, however, that the occurrence of resolution is delayed or interfered with by the administration of serum. Complications, such as empyema, may occur even after the employment of serum. In certain cases the complications have already been present when the administration of serum has been commenced; in others the complication has undoubtedly appeared after this time. From a theoretic standpoint and from what we have learned from animal experiments, we might expect that the frequency of focal infections would be increased when serum treatment is employed. Infections in susceptible animals are most likely to be focal when infection has occurred after the animals have been partially immunized, either actively or passively. Therefore if patients with severe infections which would otherwise be fatal are insufficiently treated with serum, it might be expected that, though the general infection is overcome and life is saved, focal infections might occur. Our statistics, however, do not show any increased frequency of complications among the treated cases. The non-specific treatment of cases with complications is exactly the same as though no serum had been given. Our empyema cases have all been operated upon and all except one have recovered.

Effect on Mortality.—The final test of the effectiveness of immune serum must rest on the evidence concerning the saving of lives. Although the number of cases so far treated in the Hospital of The Rockefeller Institute is not large, the results obtained have been so striking that, taken in connection with the other evidence presented, and supported, as they are, by the observations of others, they indicate almost certainly that the serum as employed by us has a marked curative effect in the treatment of pneumonia of Type I. Up to the present time 107 cases of lobar pneumonia of this type have been treated with serum, and of these but 8 have died, 7.5 per cent. When this is contrasted with the mortality of 25 to 30 per cent, as observed in our cases before we commenced serum treatment and in the cases observed elsewhere, the results obtained are definite and striking. During the past 5 years we have treated with serum every Type I case coming under our observation unless the case has been very mild or unless the patient was admitted at a time when recovery had obviously commenced. That the more severe cases were treated with serum is shown by the fact that among 105 treated cases, the blood cultures were positive before treatment was commenced in 40, or 38 per cent, while of 34 cases admitted during the same period and in which no serum treatment was given, only 2 showed positive blood cultures, or less than 6 per cent.

In the fatal cases we have included every patient that received serum, even a single dose. A brief statement as to these fatal cases may be of interest. Three of the cases were treated only a few hours before death, when they were desperately ill. One of the patients recovered from the pneumonia, but died on the 16th day following a pulmonary embolism. One patient was treated late in the disease and later suffered from numerous complications, empyema, abscesses, etc., and finally died on the 54th day from a general streptococcus infection. One patient had an extensive tuberculous involvement of both lungs, with a very small area of fibrinous pneumonia in a part of one lobe. The blood culture became negative and he apparently recovered from the pneumococcus infection, and death was probably due to the tuberculous disease. This leaves but two cases in which the serum could possibly have been of use or in which any form of specific treatment could probably have saved the lives. One of

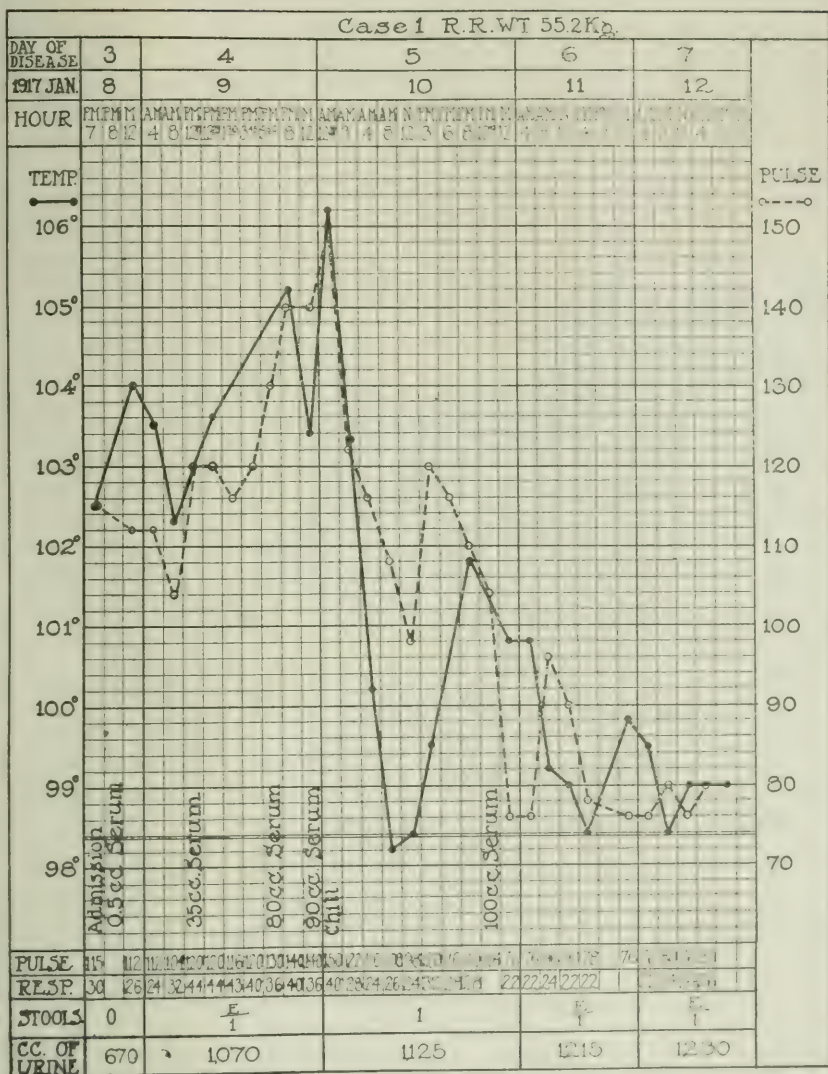
these cases was among the first ones treated by us and from our present knowledge was probably treated insufficiently. He was treated on the 5th and 6th days, dying on the latter day. The other case was one showing an extremely high grade of blood infection, 300 colonies per cc., when he was admitted on the 6th day. In spite of this, with very vigorous serum treatment, he lived until the 12th day, but finally succumbed.

The results obtained by others in small series of cases in hospitals in New York, Boston, and Pittsburgh, where the treatment could be carried out with proper attention to details, have also been definite and satisfactory. Last fall on the Mexican Border Major Nichols (18) treated 63 cases occurring among the soldiers. Of these 63 cases, 5 died, a mortality of 8 per cent. Among 18 cases with Type I infection not treated with serum, 7 died, a mortality of 39 per cent. From his experience Major Nichols concluded that "no patient with Type I infection who dies without the early intravenous administration of large doses of Type I serum can be said to have received the best treatment."

Cases Illustrating the Method of Treatment with Immune Serum.

The following abstracts of cases and temperature reactions illustrate some of the statements that have been made regarding treatment and serum reactions.

Case 1.—R. R., age 22 years, student (Text-fig. 3). This patient was admitted January 8 at 7 p.m., suffering from pneumonia involving the left lower lobe. The onset had been quite typical, with chill 48 hours before admission. He was moderately sick, temperature 102.5°F., pulse 115, respirations 30, leukocytes 31,000. Shortly after admission 0.5 cc. of horse serum was injected subcutaneously, but no previous intradermal test for serum sensitiveness had been made. The sputum was bloody; a small amount was at once injected into a mouse. The following morning tests made of the growth in the peritoneal cavity of the mouse showed that the patient was suffering from an infection with Type I pneumococcus. The blood culture taken on admission was positive, the plate showing 1 colony per cc. At 12.17 the intravenous injection of antipneumococcic serum was commenced. Although the serum was given slowly, after he had received about 35 cc. of serum he had some signs of serum intoxication, suffusion of face, some respiratory difficulty, and he vomited several times. The administration of serum was therefore discontinued. He complained of tightness in the chest



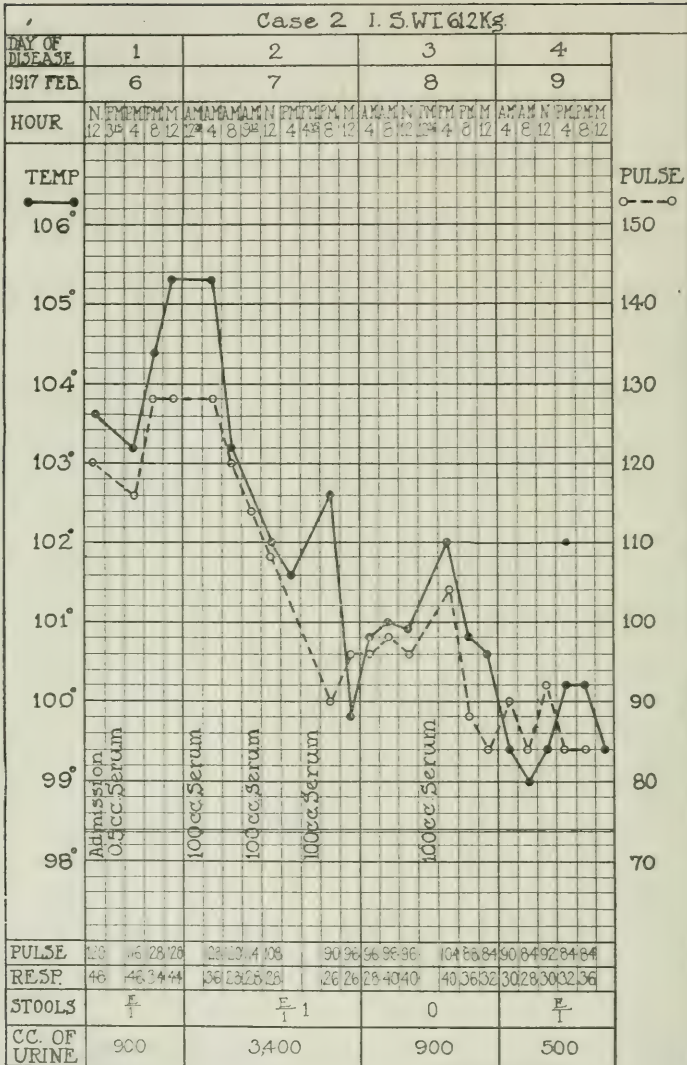
and a feeling of general weakness. 1 cc. of a 1:1,000 solution of epinephrin was injected intravenously and the patient's condition soon returned to normal, except that he sweated profusely. He was somewhat alarmed at what had taken place. His condition did not materially change during the afternoon, and at 5 p.m. serum was again administered, this time 80 cc., without any untoward symptoms. During the evening no definite change occurred in his condition. His temperature, however, had risen to 105.2°F. at 8 p.m., pulse 140, respirations 40. Therefore at 12 midnight another injection of 90 cc. of serum was made. Following this injection the temperature rose to 106.2°, pulse 150, respirations 40. He had a shaking chill during which he became cyanosed. The temperature fell rapidly during the night and at 8 a.m. it was only 98.2°, pulse 108, respirations 26. His color was good but he seemed rather weak and exhausted. During the day his condition was excellent, but the temperature gradually rose, reaching 101.8° at 8 p.m. He was then given another dose of serum, 100 cc. There was no reaction of any kind following this injection. The temperature the following morning was 99.4° and he felt quite well. There was no extension of the process in the lungs and following this the patient made a good recovery. During convalescence, however, he had quite severe serum sickness, the symptoms beginning 6 days following the last injection and some persisting for 2 weeks. There occurred persistent urticaria, stiffness and pain in the joints, edema of the face, and albumin and casts in the urine. He had some fever from January 20 to January 30, the temperature reaching 103° on January 23. However, he made a good recovery and was discharged on February 7 perfectly well, without a trace of albumin in the urine.

This patient illustrates well certain of the untoward effects of the administration of serum. It is probable that if an intradermal skin test had been made on admission, the presence of sensitiveness to serum would have been detected. As it was, he was incompletely desensitized, and he had a mild anaphylactic reaction. This, however, was not serious, and that he was completely desensitized following it is shown by the absence of reaction after the subsequent dose. Following the second large dose of serum there occurred what we have termed a thermal reaction. This was unusually severe, but very characteristic. It is possible that the following dose of serum might have been omitted or at least somewhat delayed, but it is better to have erred by giving it too soon than by delaying too long. The serum sickness was severe, one of the most severe cases we have seen. Repeated and frequent tests of the agglutinating power of the patient's serum were made in this case and, following the first dose of the horse serum, the patient's serum acquired agglutinating

power which increased following the second dose, and this increase persisted. If we had observed only this case, conclusions as to the benefit of the serum would be difficult. Judging from the fact that the blood culture was positive before the serum was given and that the patient was well, as far as the pneumonia was concerned, on the 6th day, it seems probable that the disease was shortened. In any case he recovered, and while the symptoms due to the serum were somewhat distressing, they were justified, when the results in the whole series of cases are considered.

Case 2.—I. S., age 32 years (Text-fig. 4). This patient was admitted about 8 hours following the initial chill. There were definite signs of involvement of both lower lobes and he presented all the characteristic features of acute lobar pneumonia. His temperature on admission was 103.6°F., pulse 120, respirations 48, leukocytes 35,000. He appeared seriously sick; the blood culture taken on admission was positive. Immediately after admission a specimen of sputum was injected into a mouse, and the diagnosis of the type of infecting organism, Type I, was determined within 12 hours. He was then given his first treatment. The temperature at this time was 105.2°, pulse 128, respirations 44. There was no immediate reaction following this treatment, but the next morning the temperature was lower, 103.2°, and his general condition was improved. Owing to the obviously serious nature of the infection, the treatment was continued very actively, 100 cc. being given at 9 a.m. and 100 cc. at 4.30 p.m. No marked reaction followed either of these injections, though after the injection in the evening the temperature rose slightly, but fell during the night and was 101°F. at 8 a.m. At this time he was very bright; the signs of intoxication had all disappeared. No increase of lung involvement had occurred. In spite of this he was watched carefully during the day and, as his temperature was rising, he was given another dose of serum. Following this his temperature fell. His blood was tested frequently for agglutinating power. This, which was negative before the first dose of serum, reached a high level immediately following, and this level increased with subsequent doses. He also had severe serum sickness, beginning very early, 3 days following the last dose of serum, and lasting for a week, with all the symptoms, including joint pains. On the 4th day of the serum sickness the temperature reached 103.8°. He made a good recovery and was discharged on the 24th day of the disease.

This case well illustrates the importance of early diagnosis and treatment. In this case we feel that the treatment was carried out in a perfectly satisfactory manner and, except for the serum sickness, the results were all that could be hoped for. The administration of the fourth dose on the 3rd day, we think, was of great importance in



TEXT-FIG. 4. Temperature chart, Case 2

preventing any possible recurrence of the infection and spread of the lesion.

The above cases, while favorable ones, illustrate the method of treatment and the results in a large proportion of the cases. In certain cases one or two doses of serum have sufficed; in others more have been necessary. In a number of cases no effect on the temperature curve followed the first few doses. It is therefore important that, having once commenced the serum, its administration should be actively pushed until favorable results are obtained. It is now evident that no harm can result from this procedure and, judging from our experience, much good.

PREVENTION OF PNEUMONIA.

Epidemiology.

Lobar pneumonia is generally considered to be an endemic and sporadic disease. It is habitually present among the population of certain parts of the world, showing, however, certain seasonal variations in morbidity. In spite of the fact that its incidence may reach at times a very high rate, the relation, in origin, of one case to another has never been very apparent. Diseases of typical epidemic character, on the other hand, usually spring from a limited source, attain widespread proportions often through traceable channels, and, then, after a limited time, subside, perhaps not to recur again for a period of years. Such differences between endemic and epidemic diseases, however, probably depend more upon the facility of infection and the susceptibility of the soil on which the disease is implanted than upon any essential difference in the mechanism of communication. From a public health standpoint, therefore, it is just as important to attempt to understand and control the channels of communication of an endemic as of an epidemic disease.

Lobar pneumonia was recognized as a distinct clinical entity in the time of Hippocrates. In the 16th and 17th centuries widespread epidemics are reported to have occurred, in which the character of the disease seems to have been somewhat different from that observed in modern times. In the 19th century pneumonia assumed a definitely endemic character. At present it is a common infection throughout the United States and Canada. It is frequent all over temperate Europe, and in the inhabited portions of the south temperate zone such as Australia, parts of South America, and South Africa. Although it is much less frequent in the tropics, it is often seen there among the inhabitants of the plateau regions. Small epidemics in limited communities such as military camps, schools, prisons, and on shipboard are not of infrequent occurrence, probably due to the intimate contact among individuals which such a life entails. In recent

years two epidemics of more widespread proportions have developed. One occurred during the course of construction of the Panama Canal, in which the disease affected principally the colored laborers brought to the Isthmus from the West Indies. The other took place in the Rand Mining District in South Africa. Here, as in Panama, the incidence of the disease was by far the greatest among the black boys who were imported from Central Africa to labor in the mines. These two epidemics were probably dependent upon the same factors, the great susceptibility to pneumonia of individuals coming from regions where acute respiratory infections were uncommon and the intimate contact dependent upon living in common quarters such as barracks and compounds.

The census of 1900 showed that in the United States somewhat over 10 per cent of all deaths were due to some variety of pneumonia; this, of course, includes both bronchial and lobar pneumonia, the latter being responsible for by far the greater number. Some statistics seem to indicate that the incidence of pneumonia is increasing. It is probable that this apparent increase may be due to better methods of diagnosis and recording. One may safely say, however, that the incidence of pneumonia has shown no tendency to diminish. This may be due in part to the general acceptance of the non-contagious character of lobar pneumonia and the consequent lack of adequate measures of prevention. During the same period of time such diseases as diphtheria and tuberculosis have shown a definite decrease in morbidity, and one feels inclined to ascribe this shrinkage to the widespread activity directed toward the limitation of these diseases. It is certain that until the causative factors that operate in the continued widespread incidence of lobar pneumonia have been brought to light, and until such facts as have been discovered receive practical application by Boards of Health, the continued yearly frequency and high mortality of lobar pneumonia will not be checked.

Pasteur once said that it is possible to eradicate all diseases of bacterial origin, but that first one must know the bacterial agent and record the means by which it establishes itself in the human body. In the case of pneumonia, the greatest difficulty that stands in the way of obtaining this knowledge is the paradoxical fact that a large proportion of normal individuals may constantly harbor in their

mouth secretions organisms which are apparently identical with those causing the disease. This situation, however, is not characteristic of pneumonia alone, for somewhat similar conditions are found in other diseases. For instance, the colon bacillus, an organism not far removed from the typhoid bacillus, normally inhabits the intestines of all healthy human beings, and certain cocci resembling the meningococcus, the cause of cerebrospinal meningitis, are found in the mouth secretions of healthy individuals. In these instances it has been an easy matter to separate the disease-producing bacteria from the more or less harmless saprophytes of normal mucous membranes by simple bacteriological methods such as cultural and fermentative reactions. The problem in pneumonia, however, has been a much more difficult one, since by cultural methods it is impossible to differentiate between the more harmless pneumococci and the definitely pathogenic ones. However, by the employment of the more specific immunity reactions, indications of differences between these two general groups of pneumococci have been obtained.

In view of the fact that a majority of healthy individuals harbor in the mouth an organism which has been, until the present time, indistinguishable from the pneumococcus causing pneumonia, the presumption has been fairly general that most pneumonic infections are to be considered autoinfections and that the important factor in determining the incidence of the disease is variation in susceptibility brought about by exposure or other accidental occurrences. If this assumption is true, no useful results can be hoped for from the usual preventive measures employed in other diseases which are designed to limit the spread of the infectious agent. At the Hospital of The Rockefeller Institute (R3, R51, R52) during the past few years an attempt has been made to find out whether in reality the development of the majority of cases of lobar pneumonia depends upon infection with the pneumococcus which the individual habitually carries in his mouth secretions, the immediate onset being caused by some factor which temporarily lowers resistance, or whether contact infection, either direct or indirect, plays a part of some importance. In order to throw light upon this question, the problem has been approached from several different points of attack. In view of our ability to recognize, by means of specific reactions, definite types of

disease-producing pneumococci, it has been deemed important, in addition to confirming the constancy of these types, to study also the character of the pneumococci present in the normal human mouth. In addition to the study of the differences between the pneumococci occurring during disease and those in the normal mouth, the character of the pathogenic types has been followed during the disease and convalescence, the time of disappearance from the mouth secretions of the pneumococci causing the disease noted, and the type of pneumococcus by which they were supplanted studied. The mouth secretions of a large number of healthy individuals in contact with cases of lobar pneumonia of known type have also been studied, in order to determine whether in the mouths of such individuals the disease-producing types of pneumococcus occur with greater frequency than in normal mouths and, if so, whether this may be attributed to the intimate association, the purpose being to detect the existence of actual healthy carriers of pathogenic types of pneumococcus. The type of pneumococcus present in the mouth secretions of a large number of healthy individuals in whom no history of contact with lobar pneumonia could be obtained, has also been carefully studied. The pneumococcus of the mouth has been isolated by injecting a white mouse intraperitoneally with about 0.5 cc. of saliva, which is a sufficient quantity to kill the animal if pneumococcus is present. The organism has subsequently been isolated in pure culture from the heart's blood of the mouse and the type determined by means of the agglutination test.

Types of Pneumococcus in the Mouths of Normal Persons.—In Table IX the results of a study of 297 normal individuals are given, showing the percentage of healthy people who habitually carry pneumococcus in their mouths, and also a classification of the type of pneumococcus found. For purposes of comparison the percentage incidence of the different types of pneumococcus in 454 cases of lobar pneumonia is also given (Table X).

In Table IX is presented the study of the mouth secretions of 297 individuals in whom no history of contact with an acute or recent case of lobar pneumonia could be obtained. From the mouth secretions of 116 of these individuals pneumococci were isolated, whereas from the mouth secretions of 181 no pneumococcus was obtained. Of the 116 individuals carrying pneumococcus in the mouth at

TABLE IX.

Distribution of Different Types of Pneumococcus in the Mouths of Normal Persons.

Type of pneumococcus.	Incidence.	
		per cent
I.....	1	0.8
II.....	0	0.0
IIa.....	1	0.8
IIb.....	7	5.8
IIx.....	14	11.6
III.....	34	28.1
IV.....	64	52.9
Pneumococcus present.....	116	
“ absent.....	181	
	297	

TABLE X.

Incidence of Types of Pneumococcus in Lobar Pneumonia.

Type of pneumococcus.	Incidence.	
		per cent
I	151	33.3
II	133	29.3
IIa	6	1.3
IIb	4	0.9
IIx	9	2.0
III	59	13.0
IV	92	20.3

the time of observation, the different biologic types of pneumococcus were found in the following percentage of instances: Type I was found once, an incidence of 0.8 per cent; Type II was found not at all; the Subtypes IIa, IIb, and IIx were found 22 times, a percentage incidence of 18.2. Of these Subtype II organisms, Type IIa is the least common and Type IIx the most frequent. This distribution of the variants of Type II parallels the distribution of the larger, more inclusive pneumococcus types in the normal mouth. Type III, or *Pneumococcus mucosus*, was found 34 times, a percentage incidence of 28.1. The immunologic studies of Type III have shown that this organism is one that shows little or no variation in specificity and in this it corresponds to the typical pneumococci of Type I and Type II. On the other hand, it presents a paradox

in that it is frequently found over long periods of time in the mouths of healthy individuals and, in spite of this frequency, it is the cause of the smallest number of acute cases of lobar pneumonia of the four major types of pneumococci. Type IV was encountered in normal saliva 64 times, a percentage incidence of 52.9. This type of pneumococcus is therefore the dominant type in the mouth secretions of normal individuals.

Consideration of Table X shows that in a series of 454 cases of lobar pneumonia, *Pneumococcus* Types I and II was present in 284 instances, a percentage incidence of 62.6. The dominance of these two types of organisms in disease shows a striking contrast to their almost complete absence in the mouth secretions of normal individuals. The Subtypes IIa, IIb, and IIx were responsible for 19 instances of disease, 4.2 per cent. Type III pneumococcus, despite its frequent occurrence in the normal mouth, was responsible for but 59 instances of disease, 13.0 per cent. Type IV pneumococcus, the commonest pneumococcus present in the bacterial flora of the normal mouth, was responsible for 92 of the cases of lobar pneumonia studied, or 20.3 per cent.

Comparison of these two tables shows that the pneumococci most commonly found in the mouth secretions of normal individuals give rise to a minority of the cases of lobar pneumonia. The disease produced by these organisms, with the exception of Type III, is less severe in character, indicating a lower grade of pathogenicity of these types for man. On the other hand, Types I and II cause a majority of cases of lobar pneumonia, are of high virulence for human beings, and are seldom or never found in the mouth secretions of normal individuals who have not been in intimate association with cases of lobar pneumonia. This seems to indicate that lobar pneumonia due to Types I and II does not arise from infection with a pneumococcus which is habitually carried in the mouth, but that infection with these organisms occurs from without.

Types of Pneumococcus in Sputum of Pneumonia Patients.—If all cases of lobar pneumonia were due to infection of the individual with a type of pneumococcus which was more or less constantly present in his mouth secretions, one would expect this organism to persist or at least reappear in the sputum after recovery from the disease had occurred. Therefore, a study has been made of types of pneumococcus present in the mouth during the disease, as well as those present during convalescence. After the type of organism responsible for the disease has disappeared, the type of pneumococcus present later has been determined.

In Table XI are presented the results of a study in 15 cases. These 15 cases are representative and form but a small part of the total number of cases studied.

TABLE XI.

Persistence during Convalescence of the Type of Pneumococcus Causing the Disease.

Case No.	Type of pneumococcus during height of disease.	Day of disease on which observation was made, reckoned from day of initial chill.	Type of pneumococcus during convalescence.
2,456	I	14	No pneumococcus.
		27	" "
2,467	I	31	" "
2,527	I	10	I
		24	No pneumococcus.
2,550	I	25	IIa
		32	IIa
2,571	I	9	No pneumococcus.
		16	IV
		23	IV
2,477	II	6	II
		13	IIb
		21	IIb
		29	IIb and IV
2,536	II	10	II
		17	No pneumococcus.
		25	" "
2,543	II	11	" "
		18	" "
		31	IV
2,586	II	8	No pneumococcus.
		22	" "
2,611	II	2	II
		9	IV
		16	IV
		23	IV
		30	No pneumococcus.
		37	IV
2,465	III	11	III
		13	III
		20	IIx
		27	IV
		34	IIx and IV
		40	IIx " IV

TABLE XI—*Concluded.*

Case No.	Type of pneumococcus during height of disease.	Day of disease on which observation was made, reckoned from day of initial chill.	Type of pneumococcus during convalescence.
2,488	III	4	III
		14	IV
		21	IV
		28	IV
2,550	III	14	III
		21	IV
2,689	III	7	III
		14	III
		21	III
		28	III
		35	III
2,640	III	7	No pneumococcus.
		14	" "
		21	" "
		28	" "

A study of Table XI reveals the fact that only in exceptional instances does one find in the sputum for a considerable length of time after recovery the same type of pneumococcus with which the individual was infected during the disease. The exact time at which the disease organisms disappear varies. They have disappeared as early as 7 days from the date of onset of the disease, but have been present as late as 90 days after recovery. Usually they have disappeared in 3 to 4 weeks. In a number of instances in which the disease organism has persisted for an unduly long period of time, delayed resolution or some chronic respiratory condition has been present. After the disease organism disappears, either no pneumococcus is present or the disease type has been supplanted by one of the types which are usually found in the saliva of normal individuals. These observations indicate that the type of pneumococcus responsible for lobar pneumonia in any individual is but a temporary and accidental inhabitant of his respiratory passages, and, therefore, give additional evidence that infection occurs from without. They also indicate the length of time that convalescents may be a source of infection to others in case the mode of infection is by contact.

It is seen from Table XI that Type III pneumococcus, which is known to occur frequently in the normal mouth, disappears in the majority of instances from the mouth flora of cases of pneumonia as rapidly as do pneumococci of Types I and II, which induce disease more frequently. This suggests that Type III pneumonia may also be communicable but that, owing to the frequent occurrence of this organism in the normal mouth, the lines of communication may be impossible to trace.

Pneumococcus Carriers.—Infectious diseases, aside from those spread by insects, usually spread by immediate contact, either with a person suffering from the disease, or with a convalescent, or with a healthy carrier. The importance of these modes of transmission varies with the different diseases and in many instances more than one of these mechanisms may be involved. Infection in lobar pneumonia by immediate contact with a person ill of the disease undoubtedly occurs, and we have seen a number of instances in which this mode of infection was apparently responsible. With most diseases, however, the healthy carrier may be a greater menace to the community than the infected individual, largely because of failure to recognize the carrier condition. In the epidemiology of certain diseases, notably typhoid and epidemic cerebrospinal meningitis, the importance of the carrier state is well recognized. In Table XII evidence is presented to show that a considerable percentage of persons intimately associated with patients suffering from lobar pneumonia harbor in their mouths pneumococci of the same type as those causing the disease. The persons who have been studied have been relatives or friends, especially those who have taken care of the patients. We have already shown that pneumococci of Types I and

TABLE XII.

Incidence of Carrier Condition in Healthy Individuals in Contact with Cases of Lobar Pneumonia.

Type of pneumococcus in patient.	Total no. of contacts examined.	No. of positive contacts.	
			per cent
I	160	21	13.1
II	149	18	12.1

II are not found in the mouths of normal individuals not associated with cases of pneumonia.

For purposes of comparison with Table XII, the incidence of Types I and II in the mouth flora of normal individuals unassociated with cases of lobar pneumonia is given (Table XIII).

TABLE XIII.

Incidence of Pneumococcus Types I and II in Saliva of 297 Healthy Individuals Not in Contact with Cases of Lobar Pneumonia.

Type of pneumococcus.	Incidence.	
		per cent
I	1	0.33
II	0	0.00

A study of Table XII shows that of 160 healthy individuals in contact with cases of lobar pneumonia of Type I, 21, or 13 per cent, carried in their mouth secretions at the time of examination a pneumococcus of the same type. Of 149 individuals in contact with cases of Type II pneumonia, 18, or 12 per cent, were found to carry *Pneumococcus* Type II. When these figures are compared with those from 297 healthy individuals not in contact with lobar pneumonia, in whom the incidence of Type I pneumococcus was 0.3 per cent and that of Type II was 0.00 per cent, very little doubt can be entertained that through contact with cases of lobar pneumonia due to Type I or Type II pneumococcus, healthy carriers arise with considerable frequency. In almost every instance in which a carrier of a Type I or Type II pneumococcus has been observed, the type of organism in the carrier has corresponded with that of the case with which he has been associated. Carriers of Type I and Type II pneumococcus harbor these organisms in their mouth secretions for variable periods of time, the average being from 3 to 4 weeks. At the end of this time the disease-producing type usually disappears and is replaced, if pneumococci are present at all, by one of the types of pneumococcus found in the saliva of individuals who have not been in contact with cases of pneumonia.

Types of Pneumococcus in Dust.—The possible occurrence of pneumococcus in dust has been known for some time. Little signifi-

cance, however, has been attached to this fact. In order to determine whether pneumococcus could be recovered with any regularity from dust, and what types are present, Stillman (R52) has carried out the following study. Specimens of dust were collected as follows: A piece of paper was wrapped about a small scrubbing brush, this in turn was covered with a piece of cloth, and the whole sterilized in the autoclave. The dust of the room to be studied was swept up with the sterile brush from an area about a foot square. The specimen, folded in the sterile paper, was taken to the laboratory where it was mixed with 1 to 1.5 cc. of broth, and the mixture injected into the peritoneal cavity of a white mouse. If the animal died cultures were made from the heart's blood and the type of organism responsible for its death was determined. In all, 62 specimens of dust from rooms in which no case of pneumonia was present were examined. Pneumococci were recovered from 18 of these specimens, or 29 per cent, and were absent in 44, or 71 per cent. The frequency of occurrence of the different types of pneumococcus is shown in Table XIV.

TABLE XIV.

Types of Pneumococcus Recovered from Dust of Rooms in Which Lobar Pneumonia Had Not Occurred.

Type of pneumococcus.	Incidence.	
		<i>per cent</i>
I	1	5.5
II	0	0
IIa	0	0
IIb	4	22
IIx	3	16.6
III	2	11
IV	8	44.4

From Table XIV it is seen that the types of pneumococcus usually recovered from the rooms in which healthy individuals are living correspond, as one would expect, to those found in the mouth secretions of these individuals. On the other hand, the strictly disease-producing types of pneumococcus, Types I and II, are not found unless possibly a healthy carrier is present, as was the case in the one instance given in the table.

A similar study was made of the dust of rooms in which cases of Type I or Type II pneumonia had occurred. In all, 183 specimens of dust were examined. In 109 of these pneumococcus was not found. In 74 of the specimens of dust pneumococcus was present; Table XV gives the distribution of the various types recovered.

TABLE XV.

Types of Pneumococcus Recovered from Dust of Rooms in Which Cases of Lobar Pneumonia Due to Type I or Type II Pneumococcus Had Occurred.

Type of pneumococcus.	Incidence.	
		<i>per cent</i>
I	25	33.8
II	23	31.1
IIa	0	0
IIb	2	2.7
IIx	2	2.7
III	2	2.7
IV	20	27.0

This study shows that pneumococcus is more frequently present in the dust of rooms in which lobar pneumonia has occurred than in those in which no pneumonia has existed, and under the former circumstances pneumococci of Types I and II are frequently found. Moreover, in these instances the type of pneumococcus found in the dust corresponded with the type of pneumococcus causing the disease.

Conclusions Regarding Epidemiology.—When all these observations are taken together, we have considerable evidence, contrary to the opinion previously held, that pneumonia, in a considerable proportion of the cases at least, arises by infection from without. This evidence relates mainly to the cases due to infection with Type I or Type II pneumococcus, which organisms, however, are responsible for over 60 per cent of the cases. Pneumococci of these types persist for a limited time only in the mouths of patients who have suffered from the disease, and are very rarely, if ever, present in the mouths of normal persons who have not been in immediate contact with such patients. Pneumococci are not infrequently found in dust, but those of Type I and Type II are practically never found except in the environment of persons sick of the disease or in the environment of

carriers. Possible sources of infection of those who acquire infection with pneumococci of these types, therefore, are other patients suffering from pneumonia due to the same type of pneumococci, persons who carry these organisms during convalescence, persons who have acquired the organisms by close contact with patients (healthy carriers), and dust from the immediate environment of patients or carriers.

We therefore now have evidence in regard to the mode of infection in pneumonia of these two types which affords a justifiable basis for instituting preventive measures. The conditions in regard to the other types of pneumonia are not yet sufficiently well known to justify discussion here.

General Preventive Measures.

If lobar pneumonia in a large percentage of instances is readily communicable from one individual to another, as the evidence given above seems to indicate, it is clear that public health authorities should take cognizance of this fact in their efforts to limit the widespread incidence and high mortality of this disease. Even if accurate knowledge of the exact mode of transmission were unknown, the enforcement of such precautions as are taken in the effort to control the spread of tuberculosis, epidemic cerebrospinal meningitis, and diphtheria would be justifiable. Since we have definite knowledge concerning the mode of transmission in a large percentage of the severe cases of lobar pneumonia, it is extremely important that these precautionary steps should be taken.

One of the essential measures for the success of a campaign against any communicable disease is to provide the public with all the available knowledge concerning it and to explain the dangers that each infected individual presents to those with whom he comes in contact. The first step, therefore, in the control of lobar pneumonia would seem to be the institution of an educational campaign, so that the public mind may be receptive to such measures of control as may be deemed necessary.

In order that public health boards may institute the measures necessary for the control of an epidemic disease, it has been found essential that they should obtain knowledge of the incidence of the

disease in a locality, and of the focal distribution of the cases. The only means by which this knowledge can be acquired is through compulsory notification. Certain Boards of Health now have regulations requiring notification of cases of lobar pneumonia, but these are rarely enforced, and at present the incidence of this disease can only be approximately determined by calculation from the mortality statistics. Such a measure would not, in all probability, meet with public opposition, as has developed from the attempt to bring about compulsory notification of tuberculosis or venereal disease. Because of the ease and rapidity with which the diagnosis of lobar pneumonia is made by the practitioner, the statistics obtained would probably be quite accurate. In view of the fact that only certain types of lobar pneumonia have been shown to be communicable, public health laboratories should be equipped to determine the type of pneumococcus responsible for each particular case of the disease. Additional data concerning the epidemiology of the disease would then be available.

Each case of pneumonia should be regarded as a focus for the spread of the infection and the care of each patient should include those measures which have been found serviceable in other communicable diseases. The patient should be isolated as far as possible and his communication with other individuals should be reduced to a practical minimum. Inasmuch as the pneumococcus finds its way to the outside world mainly in the secretions from the buccal and respiratory tracts, the sputum should be collected in special containers and either burned or disinfected. All utensils, handkerchiefs, bed clothing, etc., which are likely to become contaminated through contact with the mouth or otherwise should be sterilized before again being put into general use.

The studies of Stillman have shown that a large percentage of pneumonia patients contaminate the rooms which they occupy, as is shown by the large number of instances in which strictly pathogenic types of pneumococcus can be recovered from the dust of these apartments. This indicates the necessity for thorough cleansing of any room occupied by a case of pneumonia at the close of the period of convalescence. In addition, the daily cleansing of the sick room should be practised in such a way as to avoid dissemination of dust particles.

From the studies presented above it is seen that two types of carriers of pathogenic pneumococci exist: first, convalescents from the disease, and second, healthy individuals who have not contracted the disease themselves, but who have been in intimate association with cases of the disease. These individuals usually carry the pathogenic pneumococci for 3 to 4 weeks, but instances have been observed in which the pneumococci have been harbored in the mouth secretions for much longer periods. The control of healthy carriers of disease producing bacteria is one of the most difficult and perplexing problems in the regulation of infectious diseases, and one which has not as yet been satisfactorily solved. The ideal method would be to isolate and limit the contact of carriers until they no longer harbor the organisms of disease. Such a procedure unfortunately is not always practicable. In any case carriers of the strictly disease-producing types of pneumococci should be instructed to avoid promiscuous spitting, they should be told to refrain from kissing, and they should be instructed in the proper methods of disposing of any sputum or expectoration. Disinfecting mouth washes have proved of some advantage in eliminating the organisms from the nasopharyngeal secretions of meningococcus carriers, and it is possible that a similar method may prove of service in shortening the period of time in which convalescents from lobar pneumonia or pneumococcus carriers harbor the pathogenic organisms in the mouth. This procedure has not yet been carefully tried. In convalescents it is not likely to prove of great value, however, because the pneumococci are probably present in the deeper air passages not reached by the disinfectant.

It is well known that epidemics of lobar pneumonia often arise and attain considerable proportions in small communities in which people live in close association with one another, such as military barracks, labor compounds, schools, asylums, hospitals, prisons, etc. It is under these conditions that the preventive measures described above should prove of the greatest value. In the control of an epidemic disease it is necessary that prophylactic measures should be instituted coincidently with the development of the first cases, in order to prevent the wide dissemination of the virus and the consequent failure of all precautionary methods. Therefore, upon the

outbreak of pneumonia in such communities as military barracks, all patients should be immediately isolated, their contact with other individuals cut off, and a search for carriers of the disease-producing types of pneumococcus made among their associates. The carriers found should be isolated, or at least made as harmless as possible, by instituting the measures mentioned above. It is only in this way that the distribution of the pneumococcus and the resulting high prevalence of pneumonia can be prevented.

Prophylactic Vaccination.

Animal experiments have shown that it is very easy to produce active immunity to pneumococci by the injection of small doses of dead organisms, even in animals as susceptible as the mouse and rabbit, and this immunity persists for a considerable time. It is theoretically possible, therefore, to immunize men to the fixed types of pneumococci by the injection of dead cultures. The advisability of doing this in a civilian population under ordinary conditions has never been seriously considered. There are undoubtedly marked differences in the susceptibility of individuals to pneumococcus infection, just as there are differences among individuals in susceptibility to diphtheria. The so called Schick test has made the detection of individuals who are immune to diphtheria possible. If a similar method were applicable in pneumonia, so that persons either susceptible or immune to pneumococcus infection could be readily determined, the number whom it would be necessary to immunize could be greatly reduced, and probably the immunization of the entire population against infection, with certain types of pneumococci at least, would be both practical and possible.

Even in the absence of methods of differentiating the susceptible individuals from the immune, however, whenever pneumonia exists to an extraordinary degree among certain groups of individuals, and in the case of epidemics, the question of preventive inoculation should be considered. The only place, to our knowledge, where this has been attempted on a large scale has been among the workers in the mines in South Africa, where the disease prevails to a most alarming degree and causes the death of very large numbers of native workmen. These efforts were made during 1911 and 1912 under the direction of

Sir Almroth Wright (35). In spite of the many conclusions and hypotheses presented in the elaborate report of this work, no definite results as to the efficacy of the procedure were obtained. No effort was made to immunize the men against the different specific types of pneumococci; indeed no attention was paid to differences in type, and it is therefore evident that the work could only result in confusion. Later Lister (9, 10) carried on experimental studies in the South African Institute for Medical Research, both on animals and man, and established certain facts which may go far in making this method of prevention of pneumonia one of great value. He first determined the types of pneumococci causing the prevailing pneumonia, and learned that, in addition to the types found in Europe and the United States, at least one additional type exists, which is one of those most frequently encountered there. He then studied the production of active immunity to these various types in animals, and also investigated for the first time the best method of producing immunity in man. He has found that it is easier to produce immunity by intravenous than by subcutaneous injection. However, by the injection of sufficiently large doses of vaccine he has been able to produce immunity in man by subcutaneous injection. The tests of immunity employed consisted in demonstrating the presence of agglutinins and opsonins in the blood. It may be objected that these methods do not necessarily indicate active immunity. However, as far as animal studies may be applicable, it has been the experience of all investigators that animals whose blood contains these antibodies are always actively immune. We therefore believe that these tests are perfectly justifiable and reliable in judging of the presence of active immunity. As we have stated elsewhere, estimations of protective power probably give more accurate quantitative indications of immunity, but in the present circumstance such great accuracy is not important. From Lister's studies he concluded that for prophylactic purposes "three subcutaneous inoculations, at 7 days' interval, should be employed; each dose should consist of 6,000 million cocci of each group against which immunity is desired." He found that 8 months after the last inoculation his own serum still contained agglutinins and opsonins against the types of organisms injected. He has found that the reactions following the injection of even

very large doses of vaccine are slight. Our experience fully bears this out. These observations of Lister are of great value and importance. Our studies on the production of immunity in animals (R31), however, suggest strongly that more effective and rapid immunity may be produced by the frequent injection of small amounts of vaccine than by the infrequent injection of large amounts. Such a method of immunization, however, involves practical considerations, the discussion of which cannot be entered into here. The important fact is that active immunity may be produced against the specific types of pneumococci and that this immunity persists for a considerable length of time. The question of the advisability of active immunization against pneumonia is now a very acute and important one, since within a short time large numbers of susceptible men will be collected together in soldiers' camps, and it is probable that, unless preventive measures are instituted, large numbers of them will be attacked by the disease. Before preventive inoculation is undertaken on a large scale, certain questions must be answered: Is it justifiable or practicable to subject large numbers of men to the resulting inconvenience and loss of time in the hope of protecting a number of them from this serious disease? If this method is employed, how may the highest grade of immunity be produced, what should be the size of the dose, the site of injection, the frequency and number of injections, and what is the best form of antigen to be employed? A number of these problems are now being studied. The results of such a method of prophylaxis cannot, of course, be foretold with accuracy. We can only say that as far as experiments on animals are of value, the employment of this method would result in great saving of human life. A trial of this method in army camps has already been strongly recommended by Major Nichols (18) of the United States Army.

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20. Report of studies on pneumonia, *Tr. Assn. Am. Phys.*, 1913, xxviii, 606 (with Dochez).

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EXPLANATION OF PLATES.

PLATE 1.

- FIG. 1. Requirements for sputum inoculation of mouse.
- FIG. 2. Method of grasping mouse for inoculation.

PLATE 2.

- FIG. 3. Intraperitoneal inoculation of mouse.
- FIG. 4. Method of collecting peritoneal washings from sputum-inoculated mouse.

PLATE 3.

- FIG. 5. Injection of serum.



FIG. 1.]



FIG. 2.



FIG. 3.



FIG. 4.

(Avery, Chickering, Cole, and Dochez: Acute lobar pneumonia.)



FIG. 5.
(Avery, Chickering, Cole, and Doeber: Acute lobar pneumonia.)

OBSERVATIONS ON KIDNEY FUNCTION IN DIABETES MELLITUS.

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In the history of diabetes numerous theories as to the cause of the disease have been proposed, although no single one has had an anatomic basis definite enough to establish the underlying pathologic process. At present, while the etiology of diabetes is believed by most observers to be due to insufficiency of the internal secretion of the pancreas, yet pathologic anatomists have demonstrated that the kidneys of diabetic patients usually show certain well defined and characteristic lesions.

Armanni¹ was the first to point out that in diabetes there was an almost specific injury to the epithelium of the straight tubules by which they lost their cytoplasm and were transformed into hyaline-like vesicles without definite structure. Ebstein² confirmed this finding and described in coma a typical massing together of necrotic cells. Finally, Ehrlich³ proved that the peculiar hyaline degeneration described by Armanni was due to the deposition of glycogen in the cells and that the so-called "glycogenic degeneration" could be found in the majority of cases.

Albertoni and Pisenti⁴ fed rabbits and dogs with acetone, producing first albuminuria and eventually hyaline changes analogous to those already described, without, however, causing glycogenic degeneration. Trambusti and Nesti⁵ were able to produce similar lesions in phlorizinized dogs when the animals excreted appreciable amounts of acetone

1. Armanni: Quoted by Cantani, *Le diabète sucré et son traitement diététique*, 1876.

2. Ebstein: *Deutsch. Arch. f. klin. Med.*, 1881, 28, 143; 1882, 31.

3. Ehrlich: *Ztschr. f. klin. Med.*, 1883, 6, 33.

4. Albertoni and Pisenti: *Arch. f. exper. Path. u. Pharmacol.*, 1887, 23, 393.

5. Trambusti and Nesti: *Ziegler's Beitr. z. path. Anat.*, 1893, 14, 337.

in the urine. Thus it has been shown by both clinical and experimental material that the diabetic kidney has a more or less definite anatomic appearance which is comparable to that obtained in animals associated with the passage of acetone bodies from the blood into the urine. In view of these findings it is interesting that no systematic studies as to the relation of the kidney function to diabetes have been recorded. Work already reported and abundantly confirmed, however, has brought out four interesting features in this respect.

First, the diabetic kidney is at times under the influence of a diuretic. This has been shown most simply by the characteristic symptoms of polyuria and polydipsia, and somewhat more systematically by the relation between the day and night urine. Laspeyres⁶ found a nocturnal polyuria in two out of five cases of diabetes studied, and Carles⁷ in six cases. Lépine⁸ mentions that in diabetes the normal difference between day and night urine is less marked, an observation confirmed by Péhu⁹ and Külz.¹⁰

Secondly, albuminuria either constant, intermittent or terminal, is found in many cases. Thus Aldehoff¹¹ found albuminuria in 79 per cent. of 680 observations, von Noorden¹² in 21 per cent. of 650 observations, and various other writers in from 10 to 68 per cent. of their cases. While the discrepancy in figures is wide, possibly owing to the different methods of analysis employed, yet the fact remains that the diabetic kidney is abnormally prone to albuminuria.

Thirdly, edema may occur. Naunyn¹³ says that edema is not a rare occurrence in cachectic patients with severe diabetes. Williamson¹⁴ has observed anasarca in more than 5 per cent. of his patients, Frerichs¹⁵ in twenty-five out of 400 cases, and Joslin and Goodall¹⁶ in

6. Laspeyres: *Deutsch. Arch. f. klin. Med.*, 1910, **68**, 192.

7. Carles: *Province méd.*, 1906.

8. Lépine: *Le diabète sucré*, 1909.

9. Péhu: *Revue de méd.*, 1903, p. 279.

10. Külz: *Klinische Erfahrungen über Diabetes mellitus*, 1899.

11. Aldehoff: Quoted from Külz, Footnote 10.

12. Von Noorden: *Die Zuckerkrankheit und ihre Behandlung*, 1912.

13. Naunyn: *Diabetes Mellitus*, 1906.

14. Williamson: *Diabetes Mellitus and Its Treatment*, 1898, p. 227.

15. Frerichs: *Ueber den Diabetes*, 1884.

16. Joslin and Goodall: Experiments on an Ash-free Diet and Salt Metabolism, *Jour. Am. Med. Assn.*, 1908, **51**, 727.

seven cases at a time when the patients presented slight, if any, other evidence of heart or kidney disease.

Finally, the urine of patients on the verge of coma has been found repeatedly to contain masses of hyaline and granular casts, the so-called "Komazylinder" of Aldehoff.

The present paper reports studies on renal function in diabetes under varying conditions of glycosuria, hyperglycemia and acidosis. It seemed of greatest interest to study the effect on the kidney of sugar and acetone bodies because they are usually not a feature in nephritis. Moreover, it is generally believed that an increasing concentration of sugar in the blood without concomitant glycosuria is due to a specific holding back of sugar on the part of the diabetic kidney, a fact which in itself is evidence of abnormal kidney function. Under normal conditions, too, the organism depends on the kidney to regulate the acid-base equilibrium. Unless the rate of elimination of acids keeps up with their rate of production, true "acidosis" results. Thus the permeability of the kidney for both sugar and acid may well be an important factor in helping to establish the symptoms due to glycosuria and acidosis.

At present, the common tests for renal function used in heart and kidney diseases in addition to urinalysis are the phenolsulphonaphthalein test of Rowntree and Geraghty,¹⁷ some form of "test renal meal" as advocated by von Monakow,¹⁸ Hedinger and Schlayer,¹⁹ O'Hare,²⁰ and Mosenthal,²¹ estimation of nonprotein nitrogen or urea of the blood alone as advocated by Ascoli,²² Strauss,²³ and Folin,²⁴ or in relation to the simultaneous excretion of urea in the urine accord-

17. Rowntree and Geraghty: *Jour. Pharm. and Exper. Therap.*, 1909, **1**, 579.

18. Von Monakow: *Deutsch. Arch. f. klin. Med.*, 1911, **102**, 248.

19. Hedinger and Schlayer: *Deutsch. Arch. f. klin. Med.*, 1914, **114**, 120.

20. O'Hare: A Study of Salt, Nitrogen and Water Excretion in Nephritis, *THE ARCHIVES INT. MED.*, 1916, **17**, 711.

21. Mosenthal: Renal Function as Measured by the Elimination of Fluids, Salt and Nitrogen, and the Specific Gravity of the Urine, *THE ARCHIVES INT. MED.*, 1915, **16**, 733.

22. Ascoli: *Arch. f. d. ges. Physiol.*, 1901 **87**, 103.

23. Strauss: *Die Chronische Nierenzündungen in ihrer Einwirkung auf die Blutflüssigkeit und deren Behandlung*, 1902.

24. Folin and Denis: *Jour. Biol. Chem.*, 1912, **11**, 527.

ing to the method of Ambard²⁵ and McLean,²⁶ and estimation of the blood chlorid in relation to its excretion in the urine according to Ambard's laws. Comparative studies with those tests in nephritis have shown that as the renal function becomes impaired, the excretion of phenolsulphonephthalein diminishes, the nonprotein nitrogen and urea of the blood increase, the kidney is less able to excrete nitrogen and in certain instances water and sodium chlorid, and the ratio between urea in the blood and that in the urine changes so that Ambard's constant for urea becomes higher as McLean's index becomes proportionally lowered. Interesting information in regard to renal physiology has been obtained by such tests and two significant conclusions have been drawn: (1) Ambard and McLean have demonstrated that the excretion of certain substances from the blood through the kidney into the urine is carried on according to laws capable of numerical expression. (2) The kidney has selective and independent powers of excretion for several of the different urinary constituents.

For the studies reported here a series of cases of diabetes of differing severity was selected. Renal function in relation to the excretion of urea and sodium chlorid was tested by McLean's adaptation of the Ambard constant. These tests seemed sufficiently comprehensive because McLean has shown that the urea index is a good indicator of total renal function, gives practically the same information as the phenolsulphonephthalein test, and is preferable to those tests which rely on blood analysis alone, since they can be interpreted only when the intake of the substance studied is known. The chlorid excretion was determined in addition, to study renal function in more than one way. "Test meal" studies were not made because the cases differed so widely in severity as to make a common diet for all impossible. The blood and urine sugar, as well as the carbon dioxide tension of the alveolar air,²⁷ were estimated to disclose any relation be-

25. Ambard: *Physiologie Normale et Pathologique des Reins*, 1914.

26. McLean: *Clinical Determination of Renal Function by an Index of Urea Excretion*, *Jour. Exper. Med.*, 1915, 22, 212, 336; *Jour. Am. Med. Assn.*, 1916, 66, 415.

27. The carbon dioxide tension of the alveolar air was determined in the Peter Bent Brigham Hospital cases. In the others it was calculated from the capacity of the blood plasma to combine with carbon dioxide (Van Slyke: *Jour. Biol.*

tween abnormal renal function, as illustrated by ordinary tests, and glycosuria or abnormal amounts of sugar or acetone bodies in the blood. Obviously, the carbon dioxid tension of the alveolar air gave an indirect measure of the blood acetone bodies. Winterstein²⁸ and Hasselbalch,²⁹ however, have established that variations in the carbon dioxid content of the blood, and consequently of the alveolar air, are inverse to the production of nonvolatile acids. Observations to be published in another paper show that in diabetes the fall in the alveolar carbon dioxid which occurs in acidosis is more or less parallel to the increase of acetone bodies in the blood; thus the information so obtained was significant.

The tests were all made in the same fashion according to McLean's adaptation of Ambard's methods. The patients were taken for observation in the morning before breakfast to avoid the effect of feeding. One-half hour before the period began the subjects were given 150 or 200 c.c. of water and took no more fluid or food until the observation period was ended. At the beginning of the period the bladder was emptied. Thirty minutes later about 25 c.c. of blood was taken from an arm vein into a dry tube containing about 100 mg. of powdered potassium oxalate to prevent clotting. At the same time samples of alveolar air were obtained. At the end of 72 minutes³⁰ after the bladder was first emptied, the specimen of urine secreted during the 72-minute period was collected, carefully measured and used for analysis. A 72-minute period was ordinarily taken, since it is one-twentieth of 24 hours, and the calculation of the rate of excretion for 24 hours was made simple.

Chem., 1917, 30, 289), the volume per cent. of carbon dioxid bound by the plasma being multiplied by 0.69 in order to make the results numerically comparable to alveolar carbon dioxid tensions expressed in millimeters of mercury. The results from the two methods are usually alike, as has been shown by Van Slyke and by Frothingham and Walker (THE ARCHIVES INT. MED., 1916, 18, 304), although in diabetes the alveolar air sometimes indicates acidosis when the blood alkali is really normal (Stillman, Van Slyke, Cullen, and Fitz: Jour. Biol. Chem., 1917, 31, 405).

28. Winterstein: Arch. f. d. ges. Physiol., 1911, 138, 167.

29. Hasselbach: Biochem. Ztschr., 1912, 46, 403.

30. In a few cases the length of time was one or two hours. In such instances the blood was drawn in the middle of the time selected.

A portion of the blood was analyzed for urea by the method of Van Slyke and Cullen,³¹ and for sugar by the Benedict-Lewis³² method, except in a few cases when Bang's³³ micromethod was used. The remainder of the blood was centrifugalized and the plasma pipetted off. A portion of the plasma was analyzed for sodium chlorid by the McLean and Van Slyke³⁴ method, and for the combining power for carbon dioxid according to Van Slyke's³⁵ method. Alveolar air samples were collected according to the Plesch³⁶ method and were analyzed in a Haldane³⁷ gas analysis instrument. Since the blood or air was taken in about the middle of the period, it was assumed to represent the concentration in the blood for the substances whose simultaneous excretion in the urine was studied. The urine was analyzed for sugar by Benedict's³⁸ method or polarization in a few instances; for chlorids by a modified Volhard titration; and for urea and ammonia by Van Slyke and Cullen's method. The results are divided into two groups dealing with (1) the urea index, and (2) the relation of plasma chlorid to the excretion of chlorid in the urine.

In Table 1 are recorded observations on the urea index. In considering the results it is necessary to compare them with similar observations on normal individuals. McLean has published 107 such tests made according to the same methods, which serve as a good control. His tables show that the normal concentration of urea in the blood varies from 0.2 to 0.5 gm. per liter. The normal urea index varies between 80 and 200, with an average reading of 120 based on 100 tests. Any index below 80 is considered abnormal, and the degree of impairment of functional ability or damage to the kidneys becomes greater as the index gets lower. Any index above 200 is abnormal, although its significance is less certain. A high index may occur in healthy young individuals with low blood urea; it may result

31. Van Slyke and Cullen: *Jour. Biol. Chem.*, 1914, **19**, 211.

32. Benedict and Lewis: *Jour. Biol. Chem.*, 1915, **20**, 61.

33. Bang: *Der Blutzucker*, 1913.

34. McLean and Van Slyke: *Jour. Biol. Chem.*, 1915, **21**, 361.

35. Van Slyke: *Jour. Biol. Chem.*, 1917, **30**, 289.

36. Plesch: *Ztschr. f. exper. Path. u. Therap.*, 1909, **3**, 380.

37. Haldane: *Methods of Gas Analysis*, 1912.

38. Benedict: *The Detection and Estimation of Glucose in Urine*, *Jour. Am. Med. Assn.*, 1911, **57**, 1193.

TABLE 1.

The Relation of the Rate of Urea Excretion to Concentration in Blood Arranged According to the Urea Index.

$$\text{Index (I)} = \frac{\text{Gm. per 24 Hrs.} \quad \text{Gm. per Liter} \times 8.96}{\text{Wt. in Kg.} \times (\text{Blood Urea})^2}$$

Number	Subject	Weight, Kg.	24 Hour Urine, C.c.	Urea			Index I
				Gm. per Liter of Blood Ur	Gm. per Liter of Urine C	Gm. per 24 Hrs. D	
1	P. B. B. H. 6353	30.0	3,600	0.726	6.99	25.20	37
2	P. B. B. H. 6493	70.6	3,072	0.458	9.09	27.92	50
3	R. I. H. 2341	54.0	1,694	0.167	3.72	6.30	72
4	Fl.	45.0	6,000	0.360	4.16	25.00	79
5	R. I. H. 2234	50.0	2,000	0.410	12.35	24.70	93
6	R. I. H. 2480	47.0	1,680	0.273	8.11	13.62	100
7	P. B. B. H. 5921	61.7	2,880	0.267	6.76	19.47	104
8	R. I. H. 2128	40.2	1,500	0.314	10.05	15.10	108
9	P. B. B. H. 5938	40.0	4,800	0.280	3.94	18.91	108
10	P. B. B. H. 6328	68.3	1,200	0.494	30.77	36.92	110
11	P. B. B. H. 5975	64.0	1,080	0.377	25.66	27.71	138
12	P. B. B. H. 6364	64.2	1,560	0.265	12.60	19.66	139
13	R. I. H. 2280	28.5	800	0.232	9.64	7.70	140
14	P. B. B. H. 6032	48.0	1,440	0.257	10.75	15.48	144
15	R. I. H. 2680	48.0	2,000	0.262	8.88	17.76	144
16	R. I. H. 2525	47.8	4,080	0.190	3.60	14.70	145
17	L. T.	50.0	2,540	0.260	8.09	20.58	156
18	R. I. H. 2111	49.2	5,400	0.225	4.07	22.00	160
19	R. I. H. 2684	31.7	6,740	0.169	1.81	12.20	162
20	P. B. B. H. 6483	61.9	1,200	0.272	17.61	21.13	174
21	C. R.	37.3	3,460	0.162	3.11	10.75	175
22	R. I. H. 2382	46.5	1,740	0.220	9.16	15.94	192
23	P. B. B. H. 6205	62.0	780	0.212	18.23	14.22	198
24	R. I. H. 2516	87.2	2,997	0.307	15.40	46.13	198
25	R. I. H. 2414	39.8	1,280	0.165	7.11	9.09	200
26	M. L.	64.0	3,840	0.218	6.98	26.40	205
27	R. I. H. 2394	42.0	2,800	0.215	6.37	17.70	206
28	P. B. B. H. 5564	52.0	3,360	0.135	3.51	11.79	210
29	Fl.	95.4	6,000	0.230	7.44	44.64	217
30	Di.	48.7	2,200	0.192	7.38	16.24	220
31	R. I. H. 2487	50.2	1,800	0.092	3.26	5.86	223
32	R. I. H. 2469	50.4	5,000	0.160	4.17	20.85	296
33	C. A.	60.0	2,900	0.222	10.60	30.74	305
34	R. I. H. 2686	25.8	2,700	0.109	2.52	6.80	315
35	P. B. B. H. 5593	65.6	1,500	0.210	16.71	25.06	320
36	R. I. H. 2457	52.2	3,500	0.152	7.62	26.67	550
37	R. I. H. 2679	41.2	3,400	0.115	5.06	17.20	635
38*	P. B. B. H. 6482	71.3	7,320	0.240	20.65	151.16	1498

*Excluded from table of averages.

from the washing out of urea with a high fluid output; or it may occur with "vascular hypersensitiveness," according to the conception of Schlayer. Repeated indexes made on the same normal individual at different times may show such marked differences as from 87 to 196. The meaning of such variation is not defined.

Table 1 shows that twenty-one cases of diabetes, or 56 per cent. of those studied, have a urea index within normal limits. Thirteen cases, or 34 per cent., have an index above 200; four cases, or 10 per cent., have an index below 80, and must therefore be considered to have an impaired renal function. It is of interest that such a large number of cases should have a high index, especially when it is realized that the average index of those cases within normal limits is 146, which is significantly higher than McLean's normal average of 120. One reason for such findings may be the low blood urea found in several cases. Thus Case 31, with an index of 223, had a blood urea of 0.092 gm. per liter, Case 25 an index of 200, with 0.165 gm. of urea per liter of blood, and Case 28 an index of 210, with 0.135 gm. of urea per liter of blood. A more probable explanation lies in the high fluid output which occurred frequently. For instance, in the thirteen cases with an index above 200, the rate of water excretion or twenty-four hours was never below 1,500 c.c., in one case it reached 7,320 c.c., and averaged 3,770 c.c., while in the entire series the lowest output was 780 c.c. per twenty-four hours, and the average was 3,034 c.c. In McLean's normals, on the other hand, the highest fluid output encountered was 5,400 c.c., the lowest was 462 c.c., while the average was 1,738 c.c.

The patients, both normal and diabetic, had taken the same amount of fluid to drink at the same time before the period was begun. It therefore seemed that the diabetic kidney often had a rate of water elimination more rapid than normal. Since acids or sugar might possibly produce such a diuretic effect, the rate of water excretion per twenty-four hours was compared with the height of blood sugar, with the glycosuria, and with the degree of acidosis in those cases with a normal or high urea index. The results of this study are shown in Table 2.

The results studied from this point of view are inconclusive. Of the thirty-four observations, twenty-three had a fluid output above

the average normal rate of 1,740 c.c. in twenty-four hours. In this group acidosis could not be assumed to produce the polyuria, as cases with a low alveolar air showed no tendency to excrete more fluid than did those with high alveolar air. Twelve cases showed an appreciable excretion of sugar. The fluid output in these cases bore relation neither to the total excretion in twenty-four hours nor to the concentration of sugar per liter of urine. The sugar-free cases appeared to excrete water with as much ease as those with glycosuria. It is of possible significance that only four of the twenty-three cases had a blood sugar below 0.17 per cent. This observation alone might suggest that hyperglycemia was an important factor in diuresis. But against this are the eleven cases with a more nearly normal fluid output, five of which had a hyperglycemia well above 0.17 per cent.

On the whole, it appears from this series of cases that many diabetics have an abnormally high urea index. This is probably due in part to a washing out of urea through an increased output of fluid. Such polyuria does not depend on acidosis or glycosuria, but is apt to be coincident with a pronounced hyperglycemia. These findings suggest that the diabetic kidney is ordinarily hyperfunctional and hypersensitive to such a diuretic as an increased amount of sugar in the blood. They may explain in a measure the observations of earlier workers who commented on the frequency of nocturnal polyuria in the disease.

Of much greater interest both from the point of view of kidney function and diabetes are those cases with a urea index below 80, or, in other words, those cases with a definitely impaired renal function. These cases will be discussed in detail and will include certain other cases which should be placed in the same group for comparison. The cases fall into two divisions, the first consisting of one case in which the abnormal renal function was probably due to a coexistent chronic nephritis, and the second including seven cases of impending or true diabetic coma.

TABLE 2.

The Relation of the Rate of Water Excretion in Twenty-Four Hours to Sugar Excretion, and to Concentration in the Blood of Sugar or Acids (as Estimated by the Carbon Dioxid Tension of the Alveolar Air) in Those Cases of Diabetes with a Urea Index Above 80. Tabulated According to the Fluid Output.

Number	Subject	Weight, Kg.	24 Hour Urine, C.c.	Sugar			Alveolar CO ₂ , Mm.
				Gm. per Liter of Blood S	Gm. per Liter of Urine C	Gm. per 24 Hrs. D	
1	P. B. B. H. 6482	71.3	7,320	3.40	18.50	135.42	39.9
2	R. I. H. 2684	31.7	6,740	1.37	Negative	42.6
3	Fl.	95.4	6,000	2.26	7.50	45.00	35.0
4	R. I. H. 2111	49.2	5,400	2.00	Traces	37.8
5	R. I. H. 2469	50.4	5,000	1.33	Negative	38.9
6	P. B. B. H. 5938	40.0	4,800	3.18	12.50	60.00	12.8
7	R. I. H. 2525	47.8	4,080	1.56	Negative	40.2
8	P. B. B. H.	64.0	3,840	1.49	Negative	40.8
9	R. I. H. 2457	52.2	3,500	2.94	18.52	64.82	23.2
10	C. R.	37.3	3,460	2.63	32.30	111.50	15.5
11	R. I. H. 2679	41.2	3,400	2.86	Negative	43.2
12	P. B. B. H. 5564	52.0	3,360	2.30	Negative	37.3
13	R. I. H. 2516	87.2	2,997	2.63	16.40	49.14	38.8
14	P. B. B. H. 5921	61.7	2,880	2.16	Traces	31.6
15	R. I. H. 2394	42.0	2,800	2.33	22.20	62.00	20.2
16	C. A.	60.0	2,900	2.50	23.80	69.02	35.0
17	R. I. H. 2686	25.8	2,700	2.00	Negative	41.3
18	L. T.	50.0	2,540	2.56	41.60	106.00	29.6
19	Di.	48.7	2,200	4.35	34.48	75.85	36.8
20	R. I. H. 2680	48.0	2,000	3.00	16.00	32.00	20.1
21	R. I. H. 2234	50.0	2,000	2.08	Negative	38.2
22	R. I. H. 2487	50.2	1,800	2.38	Negative	42.2
23	R. I. H. 2382	46.5	1,740	3.12	23.20	40.37	35.3
24	R. I. H. 2480	47.0	1,680	4.16	Traces	33.0
25	P. B. B. H. 6364	64.2	1,560	1.37	Negative	38.2
26	P. B. B. H. 5593	65.6	1,500	1.72	Negative	42.5
27	R. I. H. 2123	40.2	1,500	2.08	Negative	36.2
28	P. B. B. H. 6032	48.0	1,440	1.03	Negative	39.1
29	R. I. H. 2414	39.8	1,280	1.67	Negative	47.6
30	P. B. B. H. 6483	61.9	1,200	2.92	31.00	37.20	33.9
31	P. B. B. H. 6328	68.3	1,200	2.56	22.00	26.40	35.1
32	P. B. B. H. 5975	64.0	1,080	1.43	Negative	38.7
33	R. I. H. 2280	28.5	800	3.12	Negative	36.9
34	P. B. B. H. 6205	62.0	780	2.82	Negative	32.5

REPORT OF CASES.

The first case, R. I. H., 2341, was a Russian woman aged 51. During a pregnancy twenty-eight years previously, the patient apparently had an attack of acute nephritis which recurred a year before entry to the hospital. Her diabetic symptoms were of two and one-half years' duration. Her physical examination was essentially negative except for an enlarged heart with an apical systolic murmur, and a blood pressure which on repeated examinations was above 190 systolic. The urine had a large trace of albumin and was without casts in the sediment. In view of the patient's history and physical examination it seemed probable that the urea index of 72 was due to a chronic nephritis, and was independent of her diabetes, which was relatively mild.

Of the coma cases, the first, C. R., was a young woman 30 years old. Her diabetes was of three years' duration, had shown a progressive, downward tendency and was accompanied by great emaciation and weakness. When seen, her physical examination was negative. Mentally she was bright and said that she was no more uncomfortable than she had been for a year. Her breathing, however, was abnormally deep, and her pulse was small and rapid. The carbon dioxid tension of her alveolar air was 15.5 mm. The urine contained acetone, diacetic acid and much sugar. There was a large trace of albumin and the sediment contained many hyaline and granular casts. Renal function tests showed a urea index of 280. The patient was treated by her own physician who reported her death about ten days later.

Three cases were seen at shorter intervals before death.

Fl. was a Russian aged 60 years, with diabetes of several years' duration. A month previously he developed a carbuncle on his neck, which was still draining, though apparently in good condition. Two days before being seen he became alarmingly sleepy and short of breath. When seen, he still could be roused, though he was evidently in a serious condition. His physical examination was negative except for his carbuncle and a diffuse bronchitis. His pulse was rapid and weak. His breathing showed marked air hunger. The urine contained much acetone, diacetic acid and sugar, had a heavy trace of albumin, and was loaded with hyaline and granular casts. Here again the carbon dioxid tension of the alveolar air was low (23.2 mm.). The urea index was 79. The patient continued to grow worse and died in a few days. No further studies on renal function could be made.

The third case, P. B. B. H., 6353, was that of a boy aged 13 years, with symptoms of diabetes of a few months' duration. The day before entry he suddenly became dyspneic and went into deep coma. His urine in addition to acetone, diacetic acid and sugar, contained a large trace of albumin and had showers of hyaline and granular casts in the sediment. The renal function tests showed a urea index of 36.5, pointing to a severe injury to his kidneys. Associated with

this was a carbon dioxid tension in the alveolar air of 12.8 mm. The patient died in a few hours.

The fourth case, R. I. H. 2787, was a girl aged 12 years. Her diabetes was of a year's duration. She first entered the hospital Nov. 16, 1916, with considerable acidosis and glycosuria which cleared up under treatment. At the time of entry her urine contained albumin and casts, her urea index was 510, her glycosuria was 27 gm. to the liter or 89 gm. in twenty-four hours, and her blood sugar was 0.31 per cent. Her alveolar carbon dioxid tension was 23.3 mm. She was discharged Dec. 23, 1916, in good condition.

She reentered March 10, 1917, in coma. According to the history she had been well until the day before, when she began to feel "short of breath" and subsequently had grown stuporous. She died in a few hours after reaching the hospital. Her urine on this admission contained more albumin than on the time before, but fewer casts. Her urea index was 34, her glycosuria was 9.52 gm. to the liter or 33.74 gm. in twenty-four hours, and her blood sugar was 0.46 per cent. Her alveolar carbon dioxid tension was 10 mm.

If these four cases are grouped together as one, it is seen that as coma developed the renal function grew worse. This was best shown by the rapidly falling urea index. It so happened, moreover, that in each case the concentration and total output of sugar were comparable, yet the blood sugar increased as the urea index fell, an observation suggesting that the kidney was becoming impermeable to sugar as well as urea.

Opportunity to inquire more specifically into kidney function in diabetic coma was afforded by three other cases which were studied for several successive days.

P. B. B. H. 6493, was a woman aged 60 years. Her history was unimportant except for a characteristic diabetic history of ten years' duration. Until a week before entry into the hospital she had been reasonably comfortable. Then for no apparent reason she had become worse and on the day of entry was nearly comatose.

She was a very plethoric, obese woman. Her physical examination was negative except as to urine and blood analyses. She lived five days. By way of treatment she was fasted and was given fluids, soup, whisky and sodium bicarbonate in large doses. The progress of her illness and its effect on renal function is shown in Table 3.

At entry the urine contained albumin and casts in addition to sugar, acetone and diacetic acid. It was evident from these signs, as well as on account of such a low carbon dioxid tension of the alveolar air (23.7 mm. tension) that she had a marked acidosis. Clinically, during the three days following admission she appeared to improve. Her kidney function, however, grew worse despite the fact

that enough alkali must have been absorbed to neutralize her acidosis in part and to cause a definite rise in the carbon dioxid tension of her alveolar air. On the morning of the fifth day the urea index was only 6. Her condition was so bad that it was impossible to obtain a specimen of alveolar air for analysis. She died within a few hours.

TABLE 3.
Case P. B. B. H., 6493.

Date	Wt. Kg.	24 Hr. Urine	Urea			Index	Sugar			Alveolar CO ₂ , Mm.
			Gm. per Liter Blood Ur	Gm. per Liter Urine C	Gm. per 24 Hrs. D		Gm. per Liter Urine C	Gm. per 24 Hrs. D	Gm. per Liter Blood S	
8/29/15	70.6	3,072	0.458	9.09	27.92	50	51.5	158.2	5.65	23.7
8/30/15	71.5	2,960	0.458	7.88	23.32	39	17.0	50.3	5.60	17.1
8/31/15	71.5	3,840	0.304	4.22	16.21	45	9.20	35.0	5.60	24.7
9/ 1/15	69.8	2,880	0.314	3.97	11.43	30	7.00	20.0	4.55	38.6
9/ 2/15	69.8	2,240	0.466	2.74	6.22	6	10.00	22.4	6.80	Not obtained

The excretion of sugar in this case is noteworthy. At entry the blood sugar was high and was accompanied by a relatively high sugar output. Under fasting the blood sugar showed a slight diminution, with a sudden rise taking place just before death. The sugar excretion on the other hand showed a persistent decrease.

P. B. B. H. 5938 is a similar case. The patient was a young woman aged 30 with an unimportant history except for diabetes. This was of two years' duration and had caused marked loss of weight and emaciation. The day before entry to the hospital she had become dyspneic and stupid. At entry she could still be roused but had pronounced air hunger. Her physical examination was negative except for her blood and urine. She died three days after entry. Her treatment in the hospital consisted in whisky and fluids by mouth. In addition she was given infusions of sodium bicarbonate and glucose. Tests for renal function gave results shown in Table 4.

TABLE 4.
Case P. B. B. H., 5938.

Date	Wt. Kg.	24 Hr. Urine	Urea			Index	Sugar			Alveolar CO ₂ , Mm.
			Gm. per Liter Blood Ur	Gm. per Liter Urine C	Gm. per 24 Hrs. D		Gm. per Liter Urine C	Gm. per 24 Hrs. D	Gm. per Liter Blood S	
6/30/15	40.0	4,800	0.280	3.94	18.91	108	12.50	60.00	3.18	17.9
7/ 1/15	40.0	4,380	0.355	4.00	17.52	62	13.00	57.00	3.72	19.5
7/ 2/15	40.0	4,016	0.374	2.50	10.00	25	23.00	92.00	5.35	12.8

In this case, as well, the urea index showed a rapidly progressing drop, which may have been hastened by the fact that the acidosis was not appreciably influenced by the alkali. The blood sugar rose but interpretation of the findings in respect to it are obscured because the patient had received glucose.

R. I. H. No. 2770, was a boy aged 9 years. His diabetes was of two years' duration. On Oct. 28, 1916, he was in fairly good condition though the urine showed some sugar and a moderate ferric chlorid reaction. He entered the hospital two days later on the verge of coma. He lived for eight days, during which time he was practically comatose. At first he was fasted and given sodium bicarbonate by mouth. On the seventh day he was given two eggs and 5 gm. of carbohydrate in vegetables. Tests for renal function are given in Table 5.

TABLE 5.—7

Date	Weight, Kg.	Urine per 24 Hrs., C.c.	Urine			Index	Sugar	
			Gm. per Liter of Blood Ur	Gm. per Liter of Urine C	Gm. per 24 Hrs. D		Gm. per Liter of Blood S	Gm. per Liter of Urine C
10/31/16	14.6	535	0.332	4.20	2.25	26.0	4.55	4.00
11/ 1/16	14.4	1,200	0.763	2.79	3.35	4.3	2.64	1.85
11/ 2/16	14.0	1,200	1.01	5.13	6.16	3.3	4.20	4.55
11/ 3/16	13.8	1,000	0.715	5.00	5.00	14.4	4.11	5.89
11/ 4/16	13.3	1,500	0.630	4.61	7.91	29.0	5.56	6.25
11/ 6/16	13.1	1,400	0.487	3.22	4.50	29.0	5.56	3.65
11/ 7/16	12.7	800	0.730	4.34	3.46	9.6	5.00	9.09

* Acetone bodies in blood and urine determined by Van Slyke's method.

In this case the urea index fell at first from 26 to 3 and then rose slightly. On the day before death it fell again. The blood sugar remained high with a comparatively small excretion of sugar in the urine. The alveolar carbon dioxide tension was low at first but rose, probably as the result of sodium bicarbonate. When the sodium bicarbonate was omitted it fell again, but returned toward normal with food. The day before death it was 33.4 mm., which would justify the conclusion that acidosis alone was not sufficient to be fatal.

Unfortunately acetone determinations were not made in the other cases. In this, however, the findings are noteworthy. The blood acetone rose to tremendously high figures while sodium bicarbonate was being given, and diminished in the blood when the drug was omitted and food was taken. On the other hand, the urinary excretion of acetone in no way kept pace with the blood concentration.

It is interesting to contrast these results with two severe cases which improved immediately under treatment and on which repeated tests were made.

R. I. H. 2680 was a woman aged 28 years with a history of diabetes developing four months before entry to the hospital. A week before, she had noticed increasing polyuria and polydipsia accompanied by dyspnea on slight exertion. Her physical examination was negative except for slight air hunger. Her urine at entry contained a trace of albumin, without casts in the sediment. There was much sugar, and a heavy diacetic acid reaction. While under observation she was fasted until she became sugar-free and was then given a diet sufficiently low in protein, carbohydrate and fat to keep her urine free from sugar.

Renal Function.

Acetone Bodies (including Beta-hydroxybutyric Acid)*			Alveolar CO ₂ , Mm.	Urinary Findings	Remarks
g. per 100 C.c. of blood A	Gm. per Liter of Urine C	Gm. per 24 Hrs. D			
50.0	8.00	4.26	15.3	Albumin and casts	259 sodium bicarbonate
40.0	7.91	9.50	20.2	Albumin and casts	69 sodium bicarbonate
60.0	7.76	9.32	30.3	Albumin and casts	159 sodium bicarbonate
70.0	7.20	7.20	34.7	Albumin and casts	29 sodium bicarbonate
68.0	4.54	6.80	26.8	Albumin and casts	
12.0	3.84	5.38	26.9	Albumin and casts	Fast broken
92.0	2.18	1.74	33.4	Albumin and casts	Fast broken

R. I. H. 2475 was a boy aged 26 months. He had been a healthy child until three weeks before entry. Then there had been a gradual onset of polyuria, polydipsia, and polyphagia. At entry the child was drowsy and extremely irritable. His physical examination was essentially negative. He was treated by fasting, after feeding for one day, and was then given carbohydrates in green vegetables to his point of tolerance. The renal function tests on the two cases are grouped together.

In both cases the urea index showed considerable variation from day to day without any progressive downward tendency. Other non-fatal cases which have been followed in similar fashion have shown variation in the urea index which, however, has usually remained well above normal. Occasional cases have been encountered which have shown temporary impairment of function. Some of these have been complicated by edema and one will be described in detail later.

These cases as a whole demonstrate two significant facts: Judged by the urea index, kidney function in diabetes is usually normal. In diabetic coma, on the other hand, pronounced renal insufficiency occurs. This is shown by a falling index which tends to become progressively lower as the severity of the condition increases. Such functional derangement may be accompanied by an increase in the blood sugar, with a lessened output, suggesting that other functions beside that of urea excretion are involved. At present the underlying cause of this complication is uncertain.

McLean has confirmed Ambard and Weill as to the laws of chlorid excretion in relation to its blood concentration and has tabulated

TABLE 6.
Case R. I. H., 2680.

Date	Weight, Kg.	24 Hr. Urine	Urea, Gm. per Liter Blood Ur	Index of Excretion I	Sugar			Alveolar CO ₂ , Mm
					Gm. per Liter Blood S	Gm. per Liter Urine C	Gm. per 24 Hrs. D	
10/16/15	48.0	2,000	0.262	144	3.00	16.00	32.00	20.1
10/17/15	48.4	2,000	0.190	230	2.70	14.30	28.60	28.7
10/18/15	49.5	6,800	0.150	142	2.62	3.00	20.40	41.4
10/20/15	49.4	2,680	0.162	210	2.33	3.77	10.10	39.5
11/3/15	11.8	1,020	0.336	414	5.70	43.48	44.35	21.8
11/4/15	11.8	1,680	0.408	256	4.65	32.26	54.20	23.6
11/5/15	11.7	600	0.186	227	2.00	3.70	2.22	27.8
11/6/15	12.2	2,300	0.228	310	1.44	Traces	Traces	23.6
11/8/15	12.2	800	0.262	230	1.54	Negative	Negative	29.4

seventy-two observations on normal individuals made according to the methods used in the present paper. McLean has found that normally the plasma chlorid varies between 5.62 and 6.25 gm. per liter according to the amount of salt ingested. There is a close agreement between the chlorid calculated in the plasma by Ambard and Weill's constants and that actually found. The maximum differences in normal individuals were, with one exception, between 0.22 above the calculated value and 0.16 below. In one case the actual chlorid was 0.38 lower than the theoretical. Under pathologic conditions relatively increased concentration of chlorid occurs in certain types of

TABLE 7.

*Rate of Chlorid Excretion (Calculated as Sodium Chlorid) to Concentration in Plasma,
Arranged According to Rate of Excretion as Modified by Concentration in Urine
and Expressed as*

$$\text{Calculated Plasma NaCl} = 5.62 + \sqrt{\frac{\text{Gm. per 24 Hrs.} \times \text{Gm. per Liter}}{\text{Wt. in Kg.} \times 4.23}}$$

Number	Subject	Weight, Kg.	24 Hrs. Urine, C.c.	Blood Urea per Liter	Index of Urea Excretion I	Blood Sugar per Liter	Alveolar CO ₂ , Mm.	Sodium Chlorid					
								Gm. per Liter Urine C	Gm. per 24 Hrs. D	Gm. per Liter of Plasma			
										Calculated	Actual	Difference	
1	P. B. B. H. 5938	40.0	4,800	0.280	108	3.18	17.9	0.20	0.96	5.67	5.56	-0.11	
2	P. B. B. H. 6493	70.6	3,072	0.458	50	5.65	23.7	0.60	1.85	5.69	5.05	-0.64	
3	P. B. B. H. 5593	65.6	1,500	0.210	320	1.72	42.5	1.70	2.55	5.73	6.35	+0.52	
4	P. B. B. H. 6353	30.0	3,600	0.736	36	4.35	12.8	0.60	2.15	5.73	6.25	+0.52	
5	R. I. H. . . .	2680	48.0	2,000	0.262	144	3.00	20.1	1.70	3.40	5.77	6.01	+0.24
6	P. B. B. H. 5921	61.7	2,880	0.267	104	2.16	31.6	2.20	6.34	5.81	6.22	+0.41	
7	P. B. B. H. 5975	64.0	1,080	0.377	138	1.43	38.7	5.10	5.51	5.83	6.31	+0.48	
8	P. B. B. H. 6205	62.4	1,440	0.212	242	2.08	41.9	4.70	6.77	5.86	5.60	-0.26	
9	R. I. H. . . .	2341	54.0	1,694	0.167	72	1.25	41.0	4.40	7.45	5.88	5.87	-0.01
10	P. B. B. H. 6364	64.2	1,560	0.265	139	1.37	38.2	5.10	7.96	5.88	5.87	-0.01	
11	P. B. B. H. 5564	52.3	3,360	0.135	210	2.30	37.3	2.70	9.07	5.88	6.02	+0.14	
12	R. I. H. . . .	2394	42.0	2,800	0.215	206	2.33	20.2	2.75	7.70	5.89	5.67	-0.22
13	R. I. H. . . .	2457	52.2	3,500	0.152	550	2.14	23.2	2.90	10.15	5.90	5.26	-0.64
14	R. I. H. . . .	2414	39.8	1,280	0.165	200	1.67	47.6	5.10	6.52	5.92	5.53	-0.39
15	C. A.	60.0	2,900	0.222	305	2.50	35.0	4.20	12.18	5.93	5.31	-0.62	
16	Gl.	70.0	2,240	84	1.10	38.1	5.50	12.32	5.93	6.03	+0.10	
17	R. I. H. . . .	2686	25.8	2,700	0.109	315	2.00	41.3	2.80	7.55	5.96	5.76	-0.20
18	R. I. H. . . .	2234	50.0	2,000	0.410	93	2.08	38.2	5.78	11.56	5.98	5.33	-0.65
19	L. T.	50.0	2,540	0.260	156	2.56	29.6	5.60	14.20	6.02	5.26	-0.70	
20	R. I. H. . . .	2516	87.2	2,997	0.307	198	2.63	38.8	7.80	23.37	6.04	6.09	+0.05
21	Di.	48.7	2,200	0.192	220	4.35	36.8	6.80	14.96	6.05	5.32	-0.73	
22	R. I. H. . . .	2684	31.7	6,740	0.169	162	1.37	42.6	2.45	16.50	6.06	5.45	-0.61
23	MI.	64.0	3,840	0.218	205	1.49	40.8	5.90	22.66	6.07	5.89	-0.28	
24	R. I. H. . . .	2382	46.5	1,740	0.220	192	3.12	35.8	8.30	14.44	6.08	5.46	-0.62
25	R. I. H. . . .	2111	49.2	5,400	0.225	160	2.00	37.8	4.30	23.20	6.10	5.88	-0.22
26	R. I. H. . . .	2679	41.2	3,400	0.115	635	2.86	43.2	5.60	19.03	6.13	5.29	-0.84
27	P. B. B. H. 6032	48.0	1,440	0.257	144	1.03	39.1	12.80	18.43	6.19	5.31	-0.88	
28	R. I. H. . . .	2469	50.4	5,000	0.160	296	1.33	38.9	6.30	81.50	6.23	5.54	-0.69

cardiac and renal disease and usually accompanies edema. Relatively low concentration of chlorid occurs in fevers and under the influence of diuretics. There is no connection between urea and chlorid functions.

As can be seen from the table, in the twenty-eight cases of this series which were studied, the plasma chlorid varied between 5.05 and 6.31 gm. per liter. In eight cases the actual plasma chlorid was higher than the calculated, while in the remaining twenty it was lower. This confirms McLean, who found a similar lowering of the plasma chlorid in the majority of his observations on twenty-eight other cases

TABLE 8.—

Date	Weight, Kg.	Diet	In- Chlorid take	24 Hr. Urine	Blood Urea	Urea Index	Sugar		
							Gm. per Liter D	Gm. per 24 Hrs. C	Blood Sugar S
6/10/16	42.0	Mixed observation diet	10.00	2,800	0.215	205	22.20	62.00	2.33
6/12/16	42.0	Fasting	10.00	2,400	0.204	91	6.20	14.90	2.68
6/13/16	42.0	Fasting	10.00	1,600	0.195	78	9.40	15.09	2.56
6/14/16	42.0	Fasting	10.00	1,900	0.180	71	6.90	13.10	3.85
6/16/16	41.6	Fasting	10.00	1,600	0.215	83	12.50	20.00	3.21
6/26/16	50.0	Fasting	10.00	4,000	0.170	62	*	*	2.32
10/ 4/16	47.8	Mixed diet	—	4,900	0.262	210	Negative	Negative	1.22

* Heavy reaction not quantitated.

of diabetes. By comparing the rate of chlorid excretion with the degree of acidosis, the urea index and the blood sugar, no interrelationship could be found. It would seem that in diabetes as well as nephritis the modes of excretion of urea and chlorid are independent.

Studies were made in three cases which may throw light on edema in diabetes.

R. I. H. 2394, was a man aged 31 years. He entered the hospital in March, 1915, with diabetes of short duration. His glycosuria responded to fasting and he was discharged sugar-free on a fairly liberal mixed diet. During his first stay in the hospital his urine was albumin-free and did not contain casts. For six months after discharge he did well. Then he grew careless, ate a diet beyond his tolerance, so that he showed sugar constantly and was on the decline. He re-entered the hospital June 9, 1916, in poor condition. His physical examination was negative. His urine contained sugar and diacetic acid. There was a trace of albumin but no casts. The carbon dioxid tension of his alveolar air was 21 mm.

His blood was strikingly lipemic. He was fasted for nine days with benefit to his acidosis and lipemia, although his glycosuria persisted. It seemed wise to interrupt his fast for four days by allowing a protein-fat diet of 1,000 calories. During this period he developed a tremendous edema so that he looked like a case of chronic nephritis. His color grew pasty, there was edema of his eyelids and genitals, as well as of his entire body.

A second fast for three days cleared his glycosuria. He was then given carbohydrates in the form of green vegetables to the point of tolerance, and finally a mixed diet of 1,700 calories containing 85 gm. of protein and 15 gm. of carbohydrate. He was discharged on this diet, sugar-free and acid-free. Repeated renal function studies were made up to and during the edema formation. They are shown in Table 8.

R. I. H., 2394.

Chlorid					Alveolar CO ₂ , Mm.	Total Blood Acetone Bodies† (Gm. Acetone per Liter)	Urinary Findings
Gm. per 24 Hrs. D	Plasma Chlorid Cl	Calculated Plasma Chlorid	Difference				
7.70	5.67	5.89	−0.21	20.2	0.842	Albumin trace; no casts	
4.17	5.40	5.80	−0.40	23.0	0.842	Albumin trace; few casts	
2.16	5.35	5.74	−0.39	26.3	0.854	Albumin trace; few casts	
2.18	5.48	5.73	−0.25	30.8	0.627	Albumin trace; no casts	
2.08	5.27	5.73	−0.46	36.2	0.590	Albumin trace; no casts	
2.00	5.58	5.70	−0.12	43.9	0.270	Albumin negative; no casts	
—	6.25	—	45.6	0.060	Albumin negative; no casts	

Blood acetone bodies determinations were made on plasma by Mariott's nephelometric method.

At the first observation the urea index was high and the patient was excreting chlorid with a plasma chlorid lower than the theoretical. As soon as fasting began the renal function became abnormal. This was shown by a falling urea index, a rising blood sugar with a lowering output, and by a marked chlorid retention and edema. Acidosis as estimated by both the alveolar carbon dioxide and actual amount of acetone in the blood diminished. It was only after the chlorid intake was restricted that the condition improved. Finally normal function returned. The effect of withdrawal of salt on the edema is shown graphically by the accompanying chart.

Two possible explanations of the condition come to mind. One is that the acetone bodies exert a specific effect on the kidneys. In support of this, there was presumably a considerable accumulation of acetone bodies in the tissues when the edema was at its height. Although the plasma bicarbonate was normal, the plasma acetone in-

creased, which, according to Marriott³⁹ and Sassa,⁴⁰ shows that the acetone content of the organs was increased as well. Another explanation is that the patient had a true nephritis which cleared up under treatment. In either event the case illustrates the importance of following the chlorid balance in cases with edema.

Another factor of importance in the development of edema in diabetes is the manner in which the body reacts to sodium bicarbonate. It has been recognized that healthy individuals as well as diabetics will develop edema after they have taken continued large doses of the drug. Widal, Lemierre and Cotoni⁴¹ followed the output of sodium chlorid in a patient who was given a known diet, and at the same time the body weight, the development of edema, and its connection with the intake of sodium bicarbonate. They were able to demonstrate that when bicarbonate was given, the excretion of chlorid decreased and edema occurred. When the bicarbonate was discontinued, an excess of chlorid was excreted and the edema disappeared. From this they concluded that bicarbonate edema was not different from other edemas and depended on chlorid retention. The two cases reported in Table 9 would tend to confirm these observers.

In each case the plasma chlorid was lowered after the ingestion of an alkali, and, coincidentally, the rate of chlorid excretion in the urine. Since normal chlorid excretion bears a definite relationship to the plasma chlorid content, any agent lowering the latter would tend to suppress the output of the former. If large amounts of sodium chlorid were taken, and were not excreted on account of a plasma chlorid lowered by alkali, edema would naturally result.

SUMMARY.

Observations on renal function were made in a series of cases of diabetes mellitus. Urea excretion was studied by the urea index of McLean. Chlorid excretion in relation to its concentration in the blood plasma was studied by Ambard and Weill's constants. In addition, observations were made on the effect on renal function of varying degrees of acidosis, hyperglycemia and glycosuria.

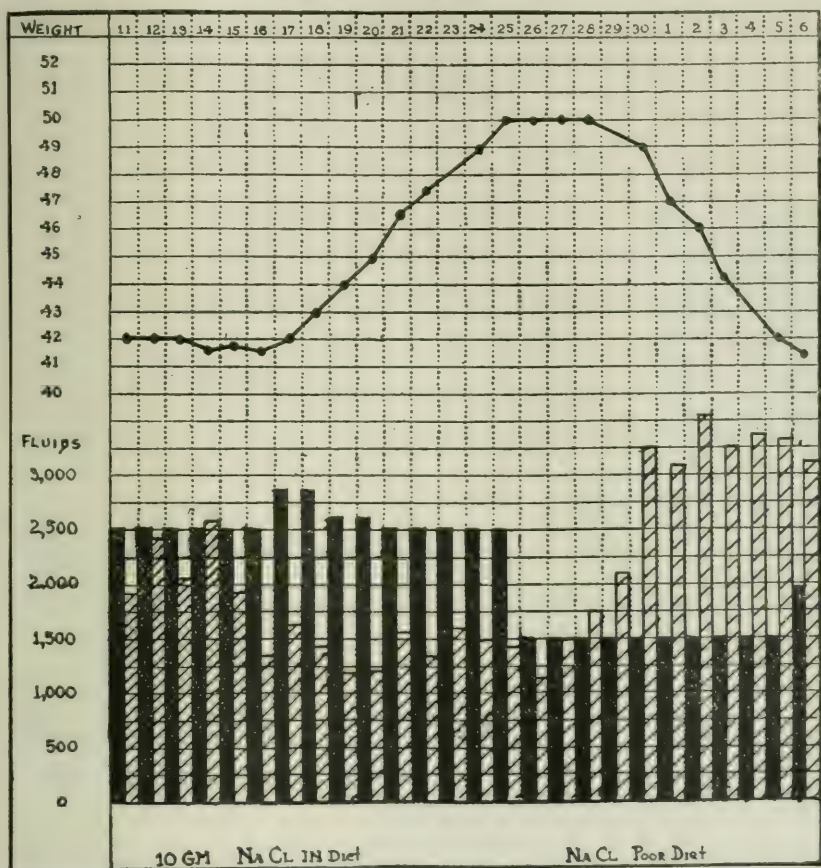
The urea index in the majority of cases tended to be normal or

39. Marriott: *Jour. Biol. Chem.*, 1914, **18**, 507.

40. Sassa: *Biochem. Ztschr.*, 1914, **59**, 362.

41. Widal, Lemierre and Cotoni: *Semaine méd.*, 1911, **31**, 325.

abnormally high. This was in part due to the rapid rate of water elimination which characterized many of the cases. Such diuretic effect was not dependent on acidosis or glycosuria, but seemed to be more or less associated with hyperglycemia.



Graphic representation of the effect of the withdrawal of salt from the diet on the edema in Case R. I. H. 2394.

The urea index in six cases of fatal diabetic coma was abnormally low. Renal function appeared to become progressively worse as the coma persisted. One patient had a pronounced accumulation of acetone in the blood plasma without a corresponding increase in excretion, and five patients showed a glycemia which seemed proportionally higher than the corresponding glycosuria. These cases suggest that

fatal diabetic coma is accompanied by impaired renal function in which more than one of the kidney's functions are involved. The cause of the complication is not known.

TABLE 9.

Sodium Bicarbonate on the Alveolar Air, Plasma Chlorid and Chlorid Excretion, Case R. I. H., 2680 Calculated Sodium Chlorid in Plasma.

Date	24 Hr. Urine Rate of Excretion	Sodium Chlorid Output Rate of Excretion in 24 Hrs.	Plasma Chlorid	Alveolar CO ₂ Mm.	Remarks
10/16/15	2,000	3.40	6.01	20.1	
10/16/15	4,272	2.56	5.68	33.6	30 gm. sodium bicarbonate
10/17/15	2,000	0.80	5.85	28.7	
10/18/15	6,800	0.68	5.37	41.4	20 gm. sodium bicarbonate
10/19/15	2,200	1.32	5.58	30.1	
10/20/15	2,680	1.34	5.26	39.5	15 gm. sodium bicarbonate
10/21/15	5,000	Traces	5.23	44.1	10 gm. sodium bicarbonate
10/25/15	4,900	2.45	5.82	39.9	

Case R. I. H., 2128

3/20/16	5,690	14.20	5.93	23.2	
3/21/16	5,000	8.40	5.99	23.0	
3/21/16	3,000	2.22	5.35	38.6	30 gm. sodium bicarbonate
3/22/16	4,400	6.50	5.89	39.0	
3/24/16	3,635	4.82	5.97	33.2	

In diabetes the blood plasma chlorid is usually lower than would be calculated from the chlorid excretion according to the formula of Ambard and Weill. This abnormality of excretion is not necessarily associated with acidosis, an abnormal urea index, the degree of glycemias or glycosuria.

Edema due to sodium chlorid retention may be encountered in diabetes. In one case it was accompanied by a falling urea index and by an increase of acetone in the blood without acidosis, as evidenced by an abnormally low alveolar carbon dioxid tension. The edema cleared up promptly when the sodium chlorid intake was restricted.

Edema following the administration of sodium bicarbonate is probably due to sodium chlorid retention, as the plasma chlorid diminishes and at the same time the excretion of sodium chlorid in the urine is lessened when the drug is given.

DETERMINATION OF TYPES OF PNEUMOCOCCUS IN LOBAR PNEUMONIA.

A RAPID CULTURAL METHOD.

By O. T. AVERY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

In view of the fact that lobar pneumonia is prevalent in military camps in the United States, and that at present it is impossible to obtain white mice in sufficient numbers for carrying out the biologic tests necessary in the determination of types of pneumococcus, a rapid cultural method dispensing with the use of these animals is now proposed.

Experience has demonstrated the importance of determining the immunologic type of pneumococcus concerned in the production of each individual case of lobar pneumonia. A satisfactory method for this consists in inoculating a white mouse intraperitoneally with a small amount of sputum, and in recovering the pneumococcus by cultural methods from the heart's blood after the death of the animal. The type of pneumococcus can be determined in this way in about forty-eight hours. When it became evident that specific type pneumococcus serum has distinct therapeutic value, it was necessary to devise a method by means of which the type of infecting pneumococcus could be determined in a shorter time. It has been found that when a mouse is injected with pneumonic sputum in which other organisms besides the pneumococcus are present, the pneumococci grow quickly, and the contaminating bacteria tend to disappear, or at least to be inhibited in their initial growth. Pneumococci and their products accumulate so rapidly in the peritoneal cavity of the infected mouse that after a few hours the bacterial exudate, collected by washing out the abdominal cavity, may be used in the performance of agglutination and precipitin tests by means of which the specific type of pneumococcus can be determined, often within from six to eight hours. Both of these methods are satisfactory, but both require the use of white mice.

It has long been known that the addition of carbohydrate, such as glucose, and of blood proteins to mediums suitable for cultivation of the pneumococcus greatly accelerates the initial growth of this organism. It is also known that bile causes solution of the pneumococcus and possesses no lytic action for other bacteria. Use has, therefore, been made of these facts in devising a method for the rapid cultivation and type determination of the pneumococcus directly from the sputum of patients suffering from lobar pneumonia. These principles are employed in the following manner:

Method.

A selected kernel of sputum, about the size of a bean, is washed, emulsified in broth, and inoculated directly into a centrifuge tube containing about 4 c.c. of special medium—glucose-blood-broth. After incubation in the water bath at 37°C. for five hours, a blood agar plate is streaked with a loopful of culture fluid for the isolation of the pneumococcus in pure culture and the subsequent confirmation of type. The red blood cells are then removed from the culture medium by slow centrifugation. Three c.c. of the supernatant bacterial suspension are pipetted into a second centrifuge tube containing about 1 c.c. of sterile ox bile and allowed to stand in the water bath at 37°C. until solution of the pneumococcus bodies has occurred, usually about twenty minutes. Five-tenths c.c. portions of the bile solution of pneumococcus are used in precipitin reaction by mixing with an equal volume of immune serums. If bile is not available, determination of the type of pneumococcus may be made directly on the bacterial suspension by macroscopic agglutination.

In carrying out the method, attention should be given to the following details:

1. *Collection of Sputum.*—Care must be exercised in obtaining a specimen of sputum from the deeper air passages, avoiding contamination with nasopharyngeal mucus and saliva. With proper supervision it is possible to obtain a small portion of sputum coughed up directly from the lung. The specimen is collected in a sterile container and sent to the laboratory at once. If, for any reason, delay should occur in the examination of the specimen, it should be kept in the ice box.

2. *Microscopic Examination*.—Direct films are made from the sputum and stained by Gram, Ziehl-Neelsen, and Hiss capsule stain. Microscopic examination of these preparations serves as a control, giving a preliminary idea of the nature of the organism present, and the cleanliness of the specimen obtained. A small portion of sputum is selected about the size of a bean, and washed through three or four changes of sterile salt solution in sterile Esmarch or Petri dishes to remove surface contamination. The selected kernel of washed sputum is transferred to a small sterile mortar, ground up, and emulsified with from 0.5 to 1 c.c. of broth, and the whole inoculated directly into 4 c.c. of the special medium.

3. *Special Medium*.—The basis of the medium is meat infusion broth¹ 0.3 to 0.5 acid to phenolphthalein, sterilized by the Arnold method, that is, twenty minutes on three successive days in order to avoid excessive heating. To each hundred c.c. of broth are added 5 c.c. of a sterile 20 per cent. glucose solution, and 5 c.c. of sterile defibrinated rabbit's blood. The final enrichment broth contains 1 per cent. glucose and 5 per cent. blood. This medium is distributed in small centrifuge tubes in quantities of about 4 c.c. each.

4. *Incubation*.—The tube of glucose-blood-broth, after inoculation with the emulsion of washed sputum, is shaken and incubated in a water bath for five hours at 37°C. This period of incubation has proved sufficient in cases thus far examined to produce an abundant growth of pneumococcus with a minimum growth of secondary organisms. The number of pneumococci present and the incubation time required in any given case can be determined by direct examination of the culture fluid.

5. *Subcultures*.—After incubation, a loopful of the sputum culture is streaked on a blood agar plate, from which a pure culture can subsequently be obtained for confirmation of the type of pneumococcus found by the rapid cultural method.

6. *Determination of Types of Pneumococcus*.—Precipitin Test: After removal of the red blood cells from the culture fluid by slow centrifugation (two minutes at low speed in order to throw down corpuscles, but not bacteria), the supernatant bacterial suspension is

1. Acute Lobar Pneumonia, Monograph 7, Rockefeller Institute, p. 25.

pipetted into a second centrifuge tube containing 1 c.c. of sterile ox bile. The bile causes solution of the pneumococcus bodies, and leaves unaffected other bacteria. Usually after twenty minutes at 37°C., solution has occurred, and is evidenced by clearing up of the bacterial turbidity of the culture fluid. Other organisms, if present, being bile insoluble, may be removed by centrifugation at high speed. The clear supernatant fluid, containing the solution of pneumococcus plus whatever specific soluble substance may have been elaborated by growth of the organism, is used in performing the precipitin test, being mixed with immune serums in quantities of 0.05 c.c. each.

Agglutination Test: If bile is not available, and if other organisms are not present in sufficient numbers to mask the specific reaction, the type of pneumococcus may be determined directly by macroscopic agglutination. The sputum culture in glucose-blood-broth is centrifuged at low speed for about two minutes to remove the red cells contained in the medium. The supernatant bacterial suspension is mixed with immune serums in quantities of 0.5 c.c. each, and incubated in water bath at 37°C. for one hour.

As various lots of immune serum differ in agglutinin and precipitin content, the dilution of serum required to assure specificity and the optimal concentration that will surely identify all type strains may vary. In view of this fact, it is well in employing the dilutions recommended² to use only serums, the agglutinin titer of which has previously been determined.

EXPERIMENTAL.

The sputums of sixty patients suffering from lobar pneumonia due to various types of pneumococcus have been studied. Pneumococcus Type I was recovered in 21 instances, Type II in 10, Type II, atypical in 5, Type III, in 4, and Type IV in 20 instances. To establish the reliability of the method, parallel determinations of the infecting type of pneumococcus have been made in every instance by mouse inoculation and by the rapid cultural method. The results by the method described have corresponded in all cases with those obtained by the intraperitoneal inoculation of mice. In three instances, however, disagreement occurred on the first examination, the mouse

2. Acute Lobar Pneumonia, Monograph 7, Rockefeller Institute, p. 23.

method yielding pneumococcus Type I, while the direct culture method showed in each case Type IV. Examination of a second specimen of the sputum in these three cases yielded results in agreement with those obtained by mouse inoculation. In the repetition of these tests, care was exercised in the selection and washing of the sputum, precautions that were purposely not taken in the first instance, in order to test the general applicability of the new method. From the results thus far obtained, therefore, it would seem that the possible inaccuracies of this method are reduced to a minimum of error by attention to the details of technic. Care in the collection of sputum from the deeper air passages with the avoidance of saliva, selection of a purulent kernel of such sputum, thorough rinsing in salt solution, and grinding in a mortar facilitate removal of surface contamination with mouth organisms, and serve to free the pathogenic type of pneumococcus from the center of the sputum mass.

Healthy persons in from 50 to 60 per cent. of instances are known to harbor pneumococci in their mouth secretions, and these organisms have been found in most instances to belong to Type IV. Normal persons who habitually carry a Type IV pneumococcus with impunity may acquire pneumonia due to infection with organisms of the more pathogenic Types I or II. This circumstance would seem at first glance to complicate the differential diagnosis of the infecting type of pneumococcus even by the most accurate method available, namely, mouse inoculation. Experience and experimental evidence have shown, however, that in such instances the disease-producing type of pneumococcus, by virtue of its greater invasiveness for animal tissues, rapidly outgrows the less pathogenic variety, and is therefore readily demonstrated in the peritoneal exudate and heart's blood of the infected mouse. On the other hand, the special medium devised for the rapid cultural method is independent of the virulence of pneumococcus, and exhibits, therefore, no selective affinity for disease-producing types. To this fact may be attributed the discrepancies described above, and on account of this fact, special precautions in technic are required. The short incubation time requisite for abundant growth of the pneumococcus in glucose-blood-broth, and the precautions already described in the selection of a suitable specimen of sputum, serve to limit to a considerable extent the growth of second-

ary organisms. The specificity of the method has been enhanced further by the utilization of the solvent action of bile, a bacterial phenomenon peculiar to the pneumococcus alone.

In many instances, the type of infecting pneumococcus has been determined more rapidly by the direct cultural procedure than by the technic of mouse inoculation. This new method, however, is presented to meet an emergency. When mice are available, the original procedure should still be employed. Only further experience can show whether or not the direct cultural method is the one of choice.

NOTES ON THE EARLY STAGES OF CHRYSOPS (DIPTERA, TABANIDÆ.)

By WERNER MARCHAND.

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Of the 34 species of chrysops known to occur in the state of New Jersey, 11 were found in Princeton; these were the following species:¹

1. *Chrysops niger* Macq. Basin, July 4, 5.
2. *Chrysops obsoletus* Wied. Greenhouse near Guyot Hall, July 8, 12; August 26.
3. *Chrysops fallax* O. S. Genetics Garden, July 6, 8.
4. *Chrysops callidus* O. S. Very common. Pond near Guyot Hall; greenhouse; Lake Carnegie; Plainsboro, June; July 4, 6, 8, 14, 15, 20.
5. *Chrysops hinei* Daecke. Near Guyot Hall, July 4.
6. *Chrysops univittatus* Macq. Carnegie Lake; Plainsboro, July 7, 8, 14, 20, 23.
7. *Chrysops lugens* v. *morosus* Wied. Basin; Genetics Garden, June 30; July 8.
8. *Chrysops montanus* O. S. Genetics Garden, July 6, 15.
9. *Chrysops striatus* Wied. Princeton July 8; August 20, 23.
10. *Chrysops vittatus* Wied. Genetics Garden, July 25.
11. *Chrysops mæchus* O. S. Princeton, July 4.

All the adult specimens obtained were ♀ ♀, except the one and only specimen of *C. mæchus*, which was a ♂. The individuals were collected partly while attacking the collector, partly from windows of the greenhouse, Guyot Hall and the vivarium of Princeton University, partly in the act of egg-laying and on flowers and bushes. One female *C. callidus* was taken in the evening of July 14, on a flower of milkweed (*Asclepias*).

¹ To these, *Chrysops uter* O. S. has to be added, which is quite common, but was overlooked in 1913, as its time of appearance does not extend into July.

The species were identified with the aid of the collections deposited in the New Brunswick Agricultural Experiment Station and the Academy of Science in Philadelphia. The writer here takes the occasion to thank Dr. Thomas Headlee, of New Brunswick, and Messrs. E. T. Cresson and Dr. Henry Skinner, of Philadelphia, for their help. For material, I am indebted to Dr. Theobald Smith for specimens of *Chrysops* from New Hampshire, and to Mr. Macy, who was in charge of the Guyot Hall greenhouse, and who collected considerable numbers of the flies. Others were collected by boys.

The Egg-Laying Habits of Chrysops.

Hart (1895) has described the egg-laying habits of *Chrysops mærens* Walker (syn. *æstuanus* Meigen). Hine (1903) describes the egg-laying habits of *C. callidus* O. S. Part of these observations have been duplicated by the writer, since at the time when the observations were made the literature was not at hand, but as our knowledge in general is still very incomplete, the following notes may add some detail to the results previously recorded.

Observations could be made on *Chrysops callidus* and one other species of *Chrysops*, which could not be fully determined, since only the eggs were found, and no specimen was caught in the act of laying. This species was probably *C. unwittatus*. It is of interest that the appearance of the egg-cluster in this species is very different from that in *C. callidus* and the other species observed.

Chrysops callidus is found ovipositing near ponds and streams on semi-aquatic weeds, chiefly in the morning hours till after 11 o'clock, from the latter part of May to the end of July. In oviposition, the females alight on a leaf or reed, head pointing downward, and proceed to lay while moving slowly in a downward direction (Plate X, fig. 1). The eggs are laid in a cohesive mass or cluster, usually on the under side, seldom on the upper side of leaves, a difference which is not marked in the case of vertically growing plants, such as *Typha*, *Sparganium*, etc. They are usually found at a height of 6 inches to 2 feet above the surface of the water, never on plants which grow at any great distance from the water. It seems that in the choice of its laying-place the female is somehow attracted by the sight of the reflecting water-surface.

The plants on which the eggs are found deposited are of a great variety, including *Pontederia cordata*, *Nuphar* spec., *Peltandra* (Aroideæ), *Typha latifolia* and *angustifolia*, *Acorus calamus*, various Gramineæ, Polygonaceæ and *Equisetum* sp. No special preference was shown for any of these, except that where *Typha* was most abundant the eggs were nearly all found on *Typha*; while where *Pontederia* was common there seemed to be more egg-masses on *Pontederia* than on any other plant. Most of the egg-masses seen in the field certainly belonged to *C. callidus*, but it is not impossible that some, while not differing in appearance, may belong to other species of *Chrysops*. The *Chrysops* egg-masses were never found on wood or on stones; the species show a decided preference for laying on plants. The egg-laying instinct is, therefore, apparently determined by two factors: the presence of water and the presence of plants growing above its surface. The egg-masses appear much less numerous in places like Plainsboro, where considerable areas are of a swampy character, evidently because the conditions for oviposition are here evenly distributed. In places, however, where the swamp-vegetation has been artificially removed, as in the case of Lake Carnegie and of the little pond near Guyot Hall (Princeton University grounds), the plant growth is developed only at the edges and it is on these alone that the conditions for oviposition are found. This, perhaps, accounts for the large numbers in which the *Chrysops* are ovipositing here and this phenomenon provides a favorable opportunity for the study of their life-history.

The time of oviposition is, in *Chrysops callidus*, the morning, from 8 to 12 o'clock; and the largest number is found ovipositing between 10 and 11 o'clock. Only very exceptionally I found *C. callidus* ovipositing at 4 o'clock in the afternoon. The habit of laying in the forenoon differentiates it sharply from the other species observed which lay in the late afternoon. The eggs are creamy white at first, and fresh egg-masses are easily recognized by their color, but in the course of a few hours the color changes first to gray then to shining black, and the majority of the egg-masses found are of this color.

The egg-masses or egg-clusters in *Chrysops callidus* are elongate in shape and much flattened, tapering at both ends, especially so at the upper end; the lower half may be abruptly truncate. Each clus-

ter consists of a single layer of eggs (as Hart has previously stated), which are regularly arranged in oblique rows: their whole arrangement being like that of shingles on a roof, with the difference that the lower eggs overlap the upper ones to about three quarters of their length; hence the arrangement is precisely the opposite of what one should expect if the intention were to prevent rain water from penetrating between the eggs. However, the whole mass, especially when a few days old, presents an almost perfectly even surface, and full protection against moisture is afforded by a thin shining outer layer or membrane, which seems to be a secretion of the eggs themselves covering the whole mass soon after oviposition is completed. It is only after piercing this membrane that individual eggs can be removed from the cluster. (See plate X, fig. 2. which, however, represents an egg-cluster of a somewhat irregular shape.)

The egg-cluster may contain from 250-300 eggs (actual counts gave 277 in one case, 260 in another). The single egg (Plate X, fig. 3), is spindle-shaped, tapering at both ends, but more so at the hind end, by which it is attached to the surface. After oviposition, it is at first semitransparent, and contains, in its upper third, an opaque whitish mass, apparently yolk. This yolk chiefly determines the whitish appearance of the cluster.

The arrangement of the eggs in the cluster is, of course, the result of the special manner in which the eggs are deposited by the female.

In the act of laying, the adult fly sits, as stated, always head downwards on the stem or leaf of the plant selected, and begins, after repeatedly trying out various places, by placing one egg about in the middle of the leaf (*Typha*); it then places a second egg on the side of this, but a little farther down. Evidently the fly first touches the first laid egg with the tip of the abdomen, and then moves the abdomen slightly downwards, by a movement which depends on or is determined by the resistance found in touching the first-laid egg. The fly then proceeds to lay, beginning from the outer edges of the egg-cluster obliquely downwards towards the lower end. Having reached this, the abdomen finds no resistance to its movements, hence it is withdrawn upwards, and a new series of eggs is laid parallel to the former, or, more frequently, the abdomen is shifted to the other side of the cluster, and here the following row is laid. Sometimes the

fly alternates regularly between the right and left side of the cluster, sometimes she may lay two or three rows of eggs on one side, ending near the middle of the cluster at the lower extremity. During oviposition, the female fly is rather quiet, and a leaf or stem may be taken from the field to the laboratory together with the egg-laying fly, as Hine has already stated. After about three-quarters of an hour the egg-mass is completed, and the fly darts off suddenly. If disturbed, however, the flies often leave in the middle of the act of oviposition. An egg-mass once abandoned is never completed, as the fly does not return to the same leaf, and evidently has no means for finding her egg-mass again, and no instinct of looking for it. Flies which in a glass jar in the laboratory had continued to lay never started laying again if they once had been disturbed and caused to leave their egg-clusters, but acted like other captive *Chrysops*, which I could never induce to deposit any eggs.

In one case only, a female, having been disturbed in the occupation of laying, and having paused for a few minutes, started again with movements of the abdomen, evidently in the intention to continue laying. However, having changed its position on the leaf but slightly, it could not reach the egg-mass any more with the tip of the abdomen; and after continuing for awhile to press the abdomen against various places on the leaf, as if in search for the egg-mass, it gave up and left. Apparently for each female it takes a long preparation until a suitable place to lay the first egg is found, while to continue the egg-laying act the presence of previously laid eggs is necessary.

Since many females of *Chrysops*, in nature, leave the egg-mass before it is completed, many of the clusters have only their upper half complete, while the lower half ends more or less obliquely truncate or "diamond-shaped" (Hine).

I have described the eggs of *Chrysops callidus*, above and recall that the white coloration of the fresh egg-clusters is due to the presence of yolk in the upper half of the otherwise semi-transparent egg. However, soon after oviposition is terminated the color of the eggs begins to change and to turn into a mottled grey, thence to shining black. This process can be easily watched under a strong lens. It is seen that the black color appears at first in the upper half of the egg on that side which corresponds to the dorsal side of the embryo

and in the shape of a symmetrical spot with several branches reaching backward about to the middle of the egg (Plate X, fig. 6). In this stage the beginnings of the embryonic development may be seen in the lower part of the egg. In about two to three hours, often sooner for in one instance only one hour was required, the entire egg has turned black. In *Chrysops callidus*, therefore, it is not possible to make any direct observations upon embryonic development. The eggs of another species which I found remain transparent and afford an excellent object for such observations.

The duration of embryonic development until hatching, is, in *C. callidus*, about five days. Eggs laid on July 6, 1916, at 11 A. M., were found hatched on July 11, at 12 o'clock, but had probably hatched in the preceding night or early in the morning. Eggs laid on July 20, at 3:30 P. M., were found hatched on July 25, at 11 P. M.

The time of hatching is almost invariably in the evening soon after sunset or later. Times of hatching recorded were 7:10 P. M., 8 P. M., on July 6, 1915; 9 P. M., on July 12; 11:20 P. M., on July 25; 8:30 A. M., on July 13; and between 11 P. M., and 9 A. M., on August 7-8. In one case at least the larvæ were observed to hatch in the morning, at 8:30. Although watched, none was seen hatching during daytime.

The act of hatching itself is not without interest. In most cases all the larvæ hatch at about the same time, each one leaving the egg through its upper pole, and the black surface of the clusters is suddenly seen covered with a whitish wriggling mass. A number of single larvæ may hatch somewhat later and the whole process may take a quarter of an hour. The larvæ are very active and decidedly thigmotactic, clinging to each other and forming masses or lumps, which soon loose their hold on the smooth surface of the cluster and drop to the ground or, under normal conditions, into the water.

It was found that it is absolutely vital for the young larvæ to reach water soon after hatching. Larvæ which were allowed to drop on a sheet of paper would crawl about for a short while, but an hour later all had died. Efforts to bring them back to life by placing them in water proved fruitless.

Reaching the water under normal conditions, the *Chrysops* larvæ at once sink to the bottom; the lumps are dissolved and each larva

moves about with a slow wriggling movement. The young larvæ measure about 1 mm. in length. They are at first positively phototropic, and the majority of them congregate on that side of the jar which is turned towards the light. This tropism is reversed after the first molt and it is evidently then that the larvæ burrow into the mud, where they seem to spend the rest of their life until pupation.

Description of the Larva of C. callidus.

The young larvæ were placed in small glass jars with a small quantity of mud and aquatic plants. Young crushed dragonfly and Chironomus larvæ were given as food. Their growth, however, was very slow and I did not succeed in keeping them alive much longer than after the first molt. This was partly due to the difficulty of furnishing them a suitable food supply and at the same time keeping the water pure and rich in oxygen, as the presence of decaying material seems fatal. I will, however, give here a description of the young larvæ, as they have never been described in any species of *Chrysops*. Concerning the larval stages of this genus, we had up to very recently only the description of the full-grown larva of *Chrysops vittatus*, by C. W. Hart (1895). It will be seen that even the very young larvæ of *Chrysops* show very marked peculiarities which permit us to differentiate them from young larvæ of *Tabanus*, at least those which I had under observation.

All Tabanid larvæ are, as we know well, comparatively uniform in structure, hence repetition in description is to some extent inevitable. The young larvæ of *Chrysops callidus* are about 1 mm. in length, elongate, tapering at both ends, with tracheæ not yet filled with air and prolegs not exerted, consequently presenting a more or less even surface. Their color is grayish white (Plate X, fig. 4). The body has twelve segments, the head is small, pointed, blackish brown in color and highly chitinized. The prolegs or parapodia are visible as small knobs on the fourth, fifth, sixth, seventh, eighth, ninth, and tenth segments, two pairs on each of these segments, forming two pairs of rows, one lateral and one ventral. On the dorsal side of the second segment a pair of black ocelli are visible which in reality lie not on the upper surface of this segment but underneath it on the

upper side of the pharynx which, with the head and mouth, is retractile. The eyes are moved forwards and backwards with the pharynx, so as to lie apparently sometimes in the second and sometimes in the third segment of the body. Similarly located at the hind end of the body and situated on the dorsal side of the tenth or eleventh segment lies the "organ of Graber," to be spoken of later, visible as two small black dots. The twelfth segment is terminated obtusely in the newly hatched larva (Plate X, fig. 4).

First Molt.—Only a few hours after hatching the larvæ, placed in water, begin to molt. The act of molting was observed repeatedly and the presence of larval skins in the water indicates that it has taken place. Plate X, fig. 5, shows an individual in the act of molting. This first molt has not been noticed in *Tabanid* larvæ, nor by Mitzmain, who has given some attention to the subject of molts.

Description of Larva After First Molt.—After the first molt the larvæ are more slender and slightly longer than before. The prolegs are now more in evidence, protruding at right angles from the body-surface, and the crawling movements become much more energetic (Plate X, figs. 7 and 8). In the structure of the head no important changes could be noticed; of these structures and of the mouth parts and antennæ, a more detailed illustration is given (Plate XI, fig. 3). Hart gives as difference between *Chrysops* and *Tabanus* larvæ the relative length of the last and second-to-the-last antennal joints; this character could so far not be verified with certainty in this young stage. A very good character which seems to separate at least those *Chrysops* and *Tabanus* larvæ which I had under observation is found in the tracheation. The main tracheal trunks of the young larva of *C. callidus* are relatively slender and narrow, being between one-eighth or one-tenth of the abdominal diameter in width, while in the young *Tabanus* larva the tracheal trunks are of much wider diameter. In the *Chrysops* larva, the diameter of the main tracheal trunks remain the same throughout their entire length, while in *Tabanus* they are much more inflated in the posterior half of the body than in the anterior (Plate XI, figs. 1 and 2).

The tracheæ are dark or shining silvery-white, according to the light, and are filled with air. The two main stems converge towards the end of the body, where they terminate in a sharp acuminate tail (fig. 8).

Description Summarized.—Body spindle-shaped, 12-segmented, slightly above 1 mm. in length; general color grayish white, semi-transparent. Head retractile; mouth parts small, dark brownish, chitinated; eyes situated on pharynx, black. Last segment ending in an acuminate tail, on the base of which are two bristles on each side. Segments 4–10 with two pairs of ventral and two pairs of lateral prolegs; the latter armed with short stiff bristles pointing backward (Plate XI, fig. 5). Intestine straight, except in the middle region of the body. Main tracheal trunks parallel in the posterior half of the body, while in the anterior half forming two large semicircular loops, ending in the region of the fourth segment, and here dividing up into small tracheæ. Chitinous surface striated as seen in Plate XI, figs. 4 and 5, Graber's organ consisting of a capsule containing only one pair of black pedunculate bodies.

Movements of the Larva.—These are carried out in the following way: the larva presses the prolegs against the surface on which it moves, then drives the main mass of the body through them by means of contractions in the posterior half of the body, while the intestine is protruded forwards and the head exerted. Then the prolegs abandon their attachment, and seem to be moved forward by the elasticity of the body to which they are attached.

Activities of the Larvæ.—The larvæ were kept in small dishes with water and some aquatic plants. In water without special care they perished in less than a week; when fed and taken care of, they lived slightly longer. As food, crushed dragonfly larvæ, mosquitolarvæ, crushed small caterpillars were given and accepted. However, their numbers always rapidly diminished. This is partly accounted for by their cannibalistic tendencies. Mitzmain states that larvæ of *Tabanus striatus*, even if other food was offered, preferred their own kind to any other food given. Another difficulty was to keep the water in small jars free from putrefaction. For this purpose green plants (*Elodea*, *Myriophyllum*) were given, but these plants decayed, and the larvæ perished. Mud seems necessary for the larvæ to burrow into, but renders their observation impossible. With a proper method, however, it should not be difficult to raise *Chrysops* larvæ, and a new attempt will be made in the coming season.

Chrysops Spec.—On July 7, 1916, 6 P. M., a *Chrysops* was seen ovipositing on the under side of a leaf of *Nuphar* (yellow pond-lily), the leaves of which often protrude above the surface of the water, on the north shore of Carnegie Lake, near the Princeton University boat-house. Unfortunately the specimen escaped, but it was undoubtedly a *Chrysops* of rather dark appearance, probably either *C. univittatus*, which is dark and next most common to *C. callidus*, or *C. lugens* v. *morosus*, or possibly *C. niger*. Specimens caught near this place belonged to *C. univittatus*. The egg-mass was collected and was very different in appearance from that of *C. callidus*. On careful search five such egg-masses were found, four on the under side of *Nuphar* leaves, one on the under side of a *pontederia* leaf nearby. As eggs of this kind were found nowhere else, it seems that the species has a predilection not only for *Nuphar* but also for the particular spot where these eggs were found.

The egg-clusters (Plate XII, figs. 1 and 2) differ from those of *Chrysops callidus* and the other species in which the oviposition has been described by the arrangement of the eggs, which are elongate as in the other species but placed with the hind end almost at right angle on the leaf-surface, one close to the other, so as to form a sort of elevated layer, the vertical thickness of which corresponds to the length of the eggs and the even surface of which is formed by the anterior ends of the eggs. The whole cluster is roundish or ovoid in outline, the sides almost right-angled, formed by the rows of eggs placed one beside the other. One of the clusters was found, by actual count, to consist of 352 eggs. The color of the freshly laid one was white, as well as in two of the others when they were found, while in the two remaining it was pale brownish. On the following day the three white egg-clusters had also assumed a brownish color, a proof that they had been freshly deposited when found. It results therefore that this species of *Chrysops* differs from *C. callidus* not only in the way the eggs are deposited and their color, but also in the time of oviposition which in this species is the early evening, before sunset.

Owing to the lack of the dark pigment which obscures the development of the eggs in *C. callidus*, in this species developmental changes may be readily observed.

Each single egg is elongate as usual in *Chrysops*, and slightly curved. It was seen that the concave side of all the eggs was turned in the same direction, that is, towards the edge of the leaf on which the cluster was found. The concave side of the egg corresponds to the ventral side of the embryo. Assuming the fly sits head-downwards while laying, that is, looking towards the base of the leaf and away from its edge, each egg is deposited in such a way that on leaving the body, the ventral surface of each egg is turned backward from the fly. On the other hand, as each egg is fastened to the leaf by its tail end, one should assume that the head-end is the last to leave the body of the fly. In this way the relative orientation of mother animal and embryo could be determined, but the material was insufficient to fully ascertain this relation.

Every single egg is seen to be somewhat shrunken on its upper pole, and its outer membrane is here contracted in three ridges meeting at the tip at about equal angles of 60° .

On the earliest stages of the development of the eggs I have no data, as the eggs were not examined before July 9, at noon, when they were almost two days old. Plate XII, figs. 3, 4, and 5, show the eggs at this stage. The embryo is plainly visible, occupying only about five-sixths of the whole length of the egg, leaving the upper one-sixth empty. The entoderm is plainly differentiated. A large mass of yolk covers the greater part of the dorsal side. Headwards on the sides are dark areas probably corresponding to the eyes which have however not yet appeared.

On the following day, July 10, at 11 P. M., these eggs were inspected again. The yolk mass had been considerably reduced, still reaching to the posterior end of the egg, and still taking almost two-thirds of the entire length. Head and pharynx have become differentiated. The eyes have appeared as two black spots on the dorsal side of the pharynx. The anterior region of the embryo has been pushed forward, and the empty portion of the egg is now less than one tenth of its entire length (Plate XII, fig. 6). When an egg is lesioned at this stage, the yolk flows out immediately as it seems to be quite liquid.

The body segments are not yet distinctly visible.

On the following day, July 11, at 5.30 P. M., the larva is seen to

fill the whole space afforded inside the egg-shell. At this stage, that is, when the embryo is exactly four days old, the final body segments begin to be visible chiefly in the anterior part of the body, and the head and pharynx is seen to be in the retracted condition, while the first body segment reaches to the anterior end of the egg. Pharynx and intestine have become more distinct; the yolk mass has shrunk further, occupying now less than one-half of the whole length, and not reaching the end of the body. The tracheæ and the organ of Graber are not yet visible in this individual (Plate XII, fig. 7). However, in a second individual from the same egg-cluster, the organ of Graber is seen already fully developed and also the tracheæ are discernible (Plate XII, fig. 8). The head is seen retracted as in the first-studied individual. Concerning the organ of Graber, it should be noted that if this organ really corresponds to a pair of modified hairs, as assumed by G. Paoli, it should always be simple in the embryo, as it is in fact in *Chrysops*. However, it seems that in the young *Tabanus* conditions are somewhat different. The body segments in the larva (Plate XII, fig. 8) are seen fully marked; the hind end of the body is slightly longer than the space afforded to it, and the last segment is curved in order to find room in the egg. All through these stages we notice a gradual increase in length of the whole embryo in the egg-shell, with the result that even with head retracted within the first segments, and with tail curved backward, it fills the whole available space. This may be of considerable importance in the act of hatching from the egg, as it enables the embryo to burst the egg-shell by simply stretching its body. On Plate XII, figs. 11 and 12, these movements are illustrated.

On the following day, July 12, 4 P. M., the eggs had not yet hatched. However, if the eggs are placed on a slide, a slight pressure of the coverglass is sufficient to cause them to leave the egg with vigorous movements. The young larva (Plate XII, fig. 9) is seen to be a typical *Chrysops* larva, eleven-segmented, with seven pairs of lateral prolegs which are not yet protruded, with two slender tracheal trunks forming loops in the anterior part of the body. The head is retracted and remains so in the larvæ which were caused to hatch prematurely. The yolk mass is still present but takes less than one-third of the whole body length. The lower part of the intestine shows regular windings similar to those seen in *C. callidus*. The organ of

Graber is distinctly seen immediately behind the dorsal bloodvessel, but not connected with it (Plate XII, fig. 10); the space containing the two pedunculate bodies¹ seems to be surrounded by a double capsule.

This egg-cluster perished in consequence of having been placed in too damp conditions, which caused the leaf to which it was attached, to disintegrate. The leaves of *Nuphar* decompose with great rapidity when detached from the plant.

However, eggs from another cluster of the same species were found hatched on July 9, at 11 A. M. The larvæ were positively phototropic, and much like those of *C. callidus*. They were kept alive for one week in a Petri dish with *Ceratophyllum* and crushed *Agrion* larvæ, but after this period all died.

A third type of egg-cluster was found several times on leaves of *Sagittaria* and *Nuphar*, and of which I can not say whether it belongs to a *Chrysops* or a very small species of *Tabanus*. These egg-clusters are obliquely conical, brown in color; the eggs of which they consist, are elongated as in the other species.² Larvæ which hatched from one of these clusters had more the appearance of *Chrysops* than of *Tabanus*, this (1) because of their small size, (2) because of the tracheæ being narrow as in *Chrysops callidus* and the other species spoken of. They were evidently aquatic but perished within a few days.

EXPLANATION OF FIGURES.

PLATE X.

FIG. 1. Female of *Chrysops callidus* O. S., ovipositing on a leaf of *Typha*. Drawn from life.

FIG. 2. Egg-cluster of *Chrysops callidus*, somewhat abnormally shaped.

¹ It has not been ascertained whether these bodies were pedunculate or not, as the organ of Graber was not known to me then, and no descriptions were at hand. I use here the term "pedunculate bodies" as used by the authors on the subject, for the black bodies readily seen in the capsule which in *Tabanus* have been shown to be "pedunculate."

² I notice in this connection J. S. Hine's statement that the egg-masses of *Chrysops celer* consist of several layers of eggs, being brownish in color. Presumably, then, the egg-masses spoken of belong to *C. celer* or to a related species.

FIG. 3. Single egg of the same species. Upper half white (tip translucent), lower half transparent.

FIG. 4. Newly-hatched larva of *Chrysops callidus*. Prolegs still retracted.

FIG. 5. Young larva of same species, in first molt. Drawn from life.

FIG. 6. Egg of *Chrysops callidus*, a few hours after oviposition, showing pigment developing symmetrically.

FIG. 7. Larva of *Chrysops callidus* after first molt. Ventral side, showing ventral and lateral prolegs.

FIG. 8. Same larva (slightly more extended). Dorsal side, showing tracheal trunks and Graber's organ.

PLATE XI.

FIGS. 1 and 2. Young larvæ of *Chrysops callidus* and *Tabanus atratus*, to illustrate the difference in the tracheal system.

FIG. 3. Head and first segment of young larva of *Chrysops callidus*, magnified. Note the long terminal joint of the antennæ. The first joint, which is very short, was not noticed when the drawing was made.

FIG. 4. Posterior end of newly-molted larva. Dorsal view. Graber's organ, *sk.*, shed skin, still adhering.

FIG. 5. Posterior end of newly-molted larva. Ventral view, showing anus, stigmatal spine and bristles.

PLATE XII.

FIG. 1. Egg mass of *Chrysops* spec. on underside of *Nuphar* leaf (turned upside down). Lateral view.

FIG. 2. Same egg-mass seen from above.

FIGS. 3, 4 and 5. Dorsal, lateral and ventral view of two-day-old egg of *Chrysops* spec. Note the empty space above the embryo.

FIG. 6. Egg and embryo of the same species, three days old. The eyes have appeared. The yolk is diminished in quantity.

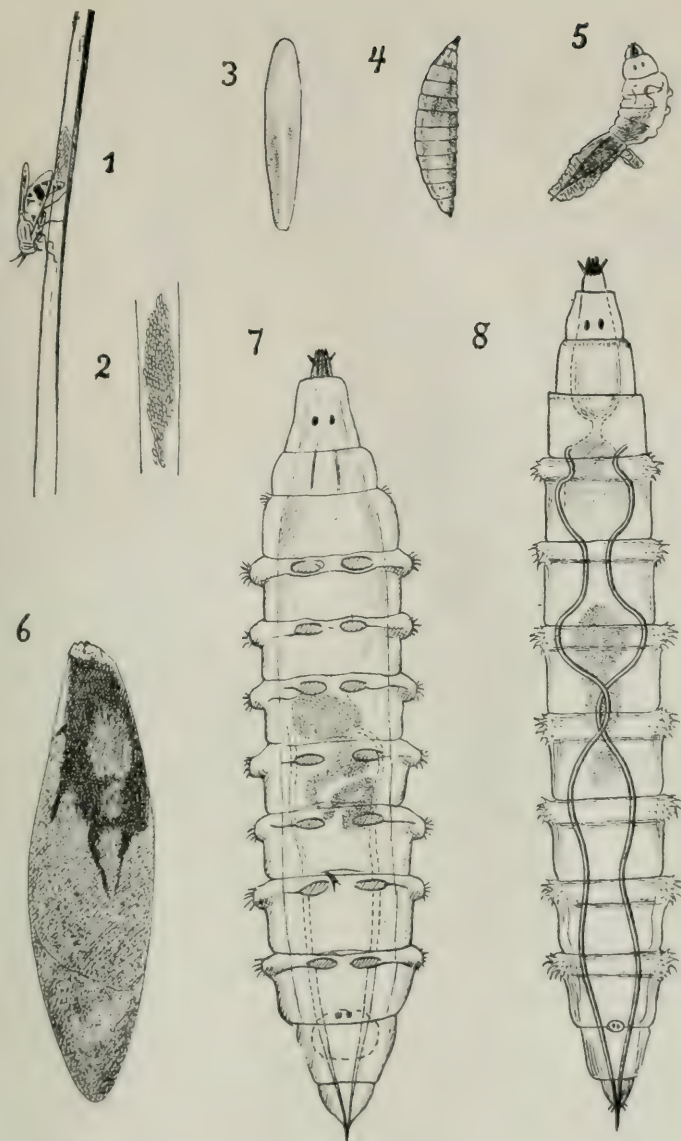
FIG. 7. Egg with embryo of same species, four days old.

FIG. 8. Egg from same egg-cluster, four days old, but developed a little farther. Body segments and Graber's organ developed.

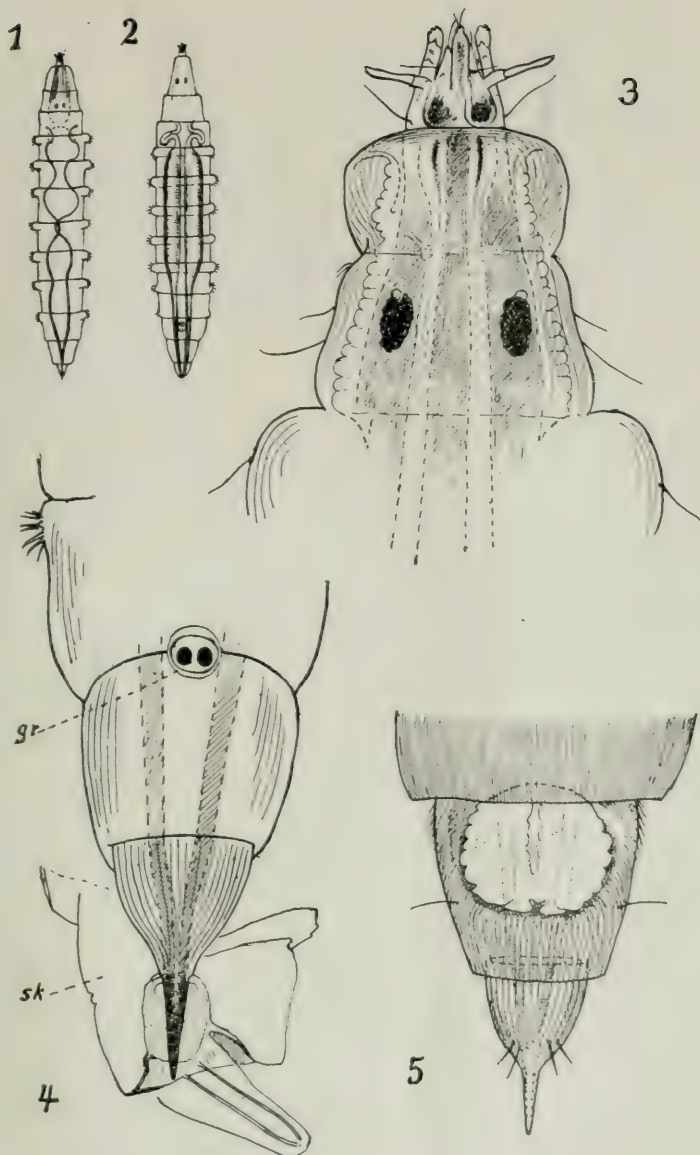
FIG. 9. Newly-hatched larva of *Chrysops* spec. Head retracted and prolegs not yet exserted.

FIG. 10. Graber's organ and posterior part of dorsal vessel of same larva, magnified.

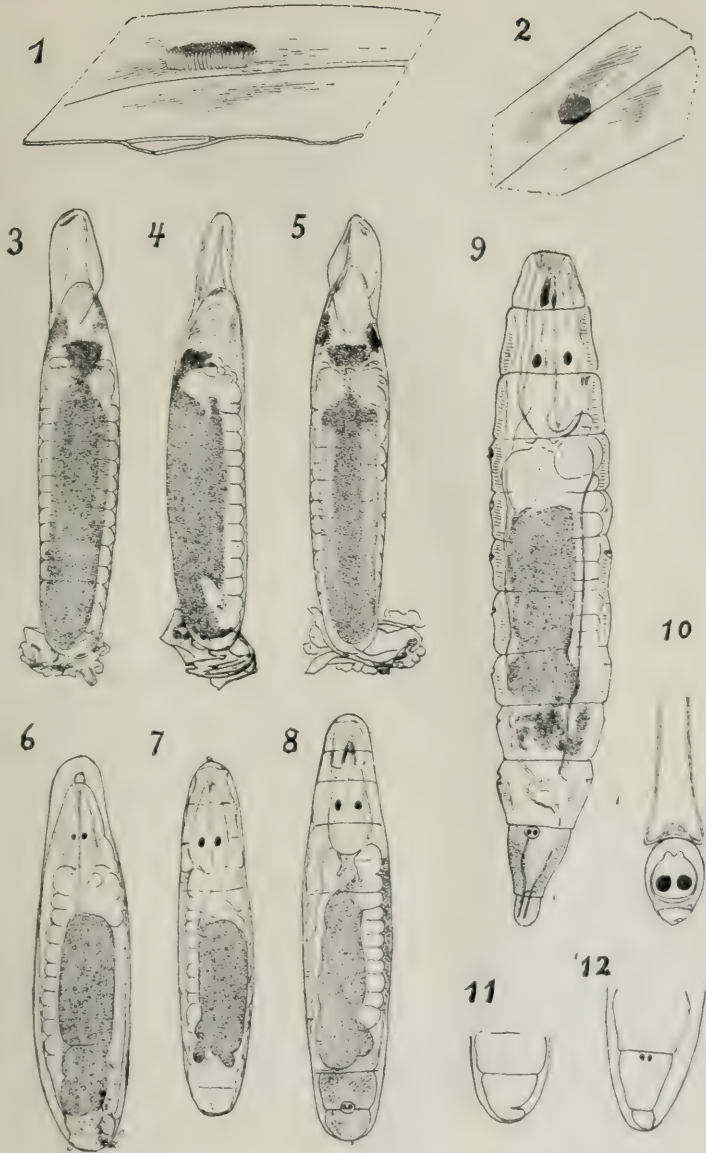
FIGS. 11 and 12. Movements of the posterior part of the body of the larva before hatching.



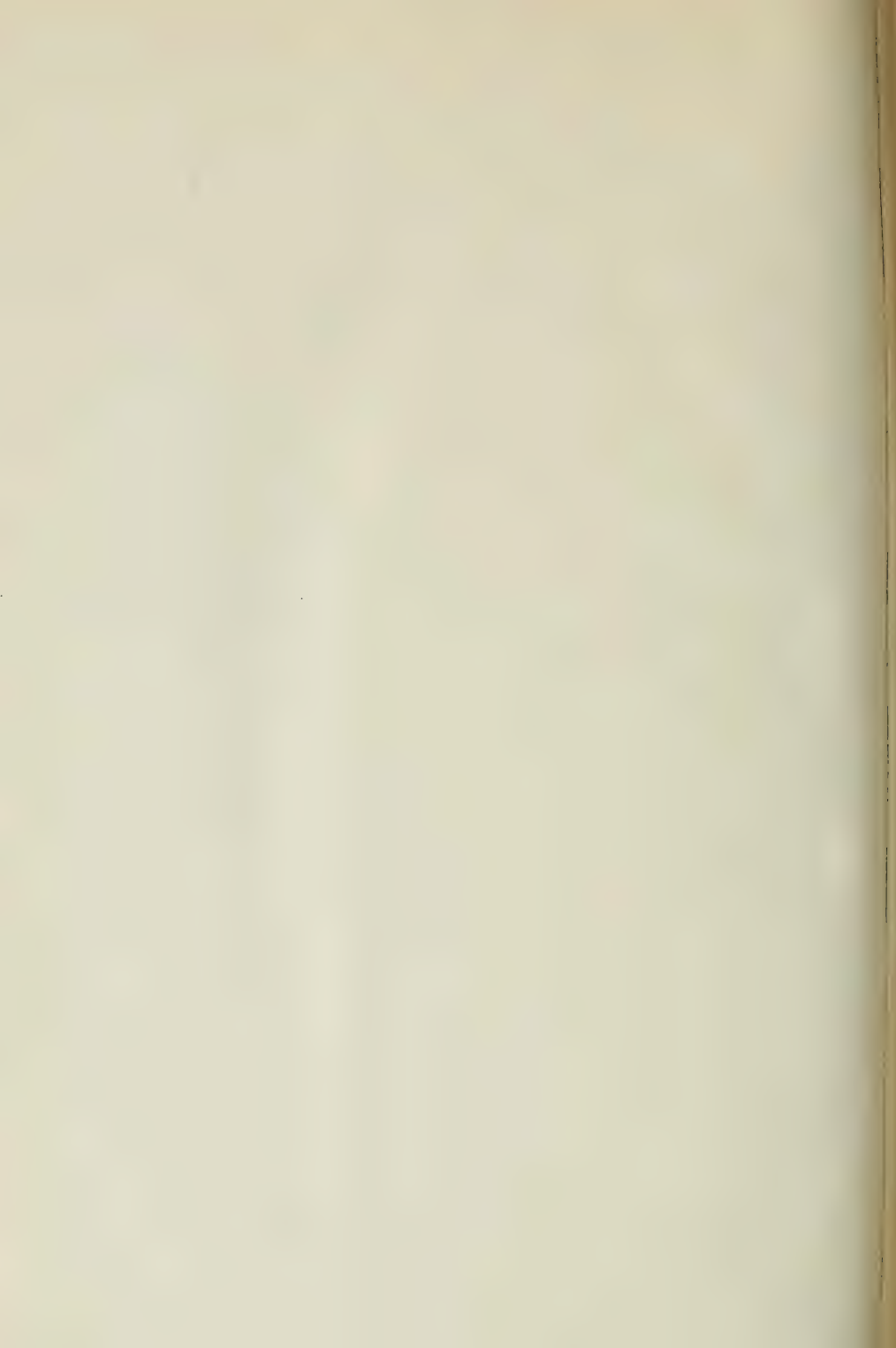
Chrysops.



Chrysops.

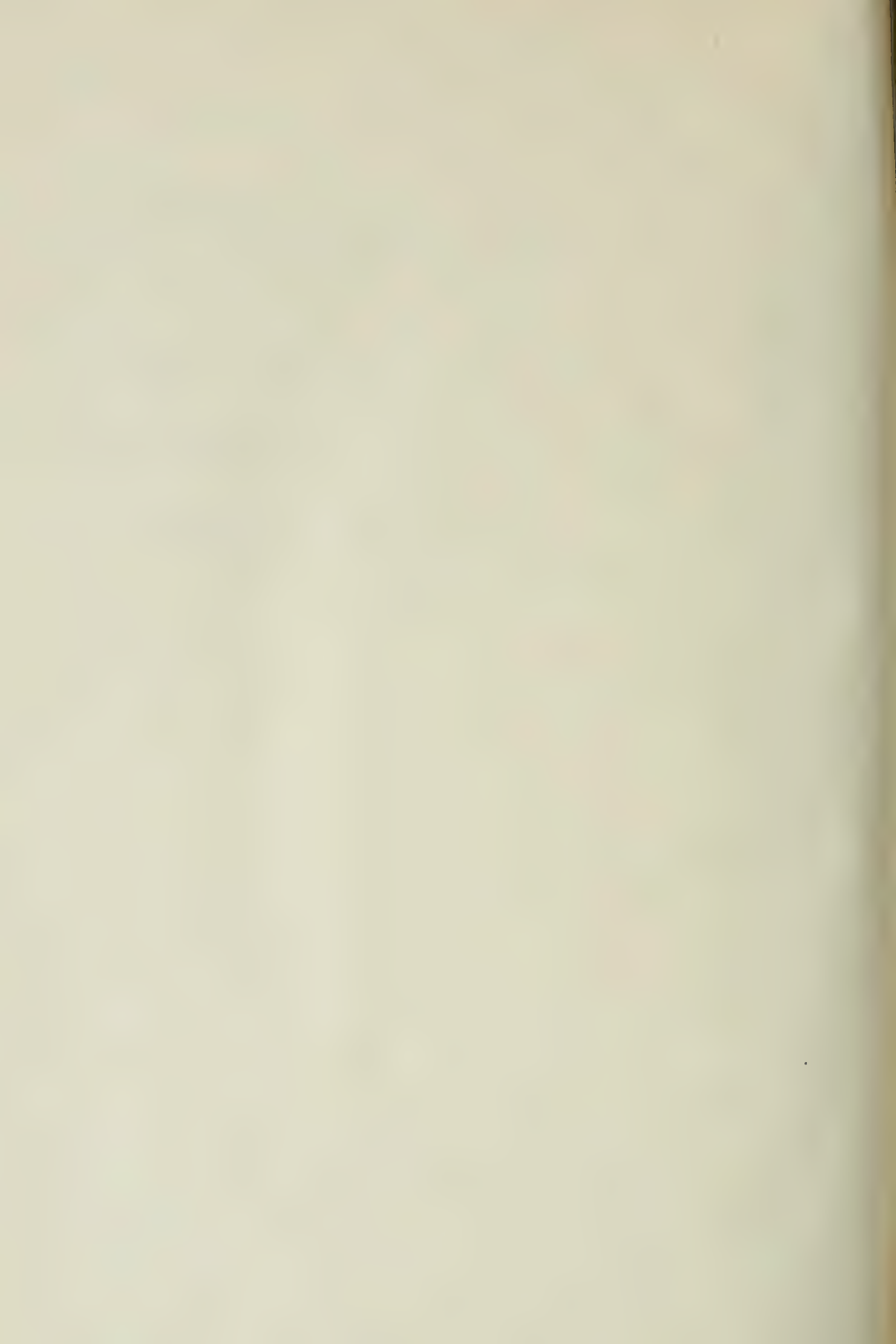


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